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POTASSIUM ION TRANSPORT AND ADENOSINE TRIPHOSPHATE SYNTHESIS IN PEA COTYLEDON MITOCHONDRIA

by

WILLIAM MURRAY HAMMAN

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

"POTASSIUM ION TRANSPORT AND ADENOSINE TRIPHOSPHATE SYNTHESIS IN PEA COTYLEDON MITOCHONDRIA"

submitted by William Murray Hamman, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

In order to examine relationships between ion transport and adenosine triphosphate (ATP) synthesis, it was necessary to isolate large amounts of mitochondria that were in as good a condition and as pure as possible. For this purpose a method was developed for zonal ultracentrifugation of a cell homogenate from etiolated pea cotyledons. Sucrose density gradient centrifugation in a Ti-XIV zonal rotor was used to obtain a large amount of mitochondria in a short time. Electron microscopy and enzymatic analysis indicated high purity in the mitochondrial fraction.

Methodology for the measurement of K^+ transport in the isolated mitochondria was developed. The advantages of the liquid-membrane electrode for measurement of K^+ far outweigh those of the glass K^+ selective electrode. Valinomycin was added to initiate K^+ uptake. No interference of the added valinomycin with the K^+ electrode was detected.

The induced K^+ uptake in mitochondria was found to be energy-dependent and greatly influenced by conditions in the ambient solution. The extent of K^+ uptake and the rate were dependent on the presence of anions and respiratory substrate and occurred only with mitochondria displaying good respiratory parameters.

By simultaneous monitoring of oxygen concentration, it was observed that when mitochondria deplete the solution of oxygen rapid efflux of K^+ occurred from the mitochondria into the surrounding medium. Addition of H_2O_2 caused rapid aeration of the solution through the action of catalase. After addition of H_2O_2, K^+ uptake again occurred at the same rate and extent as the initial K^+ uptake.

Investigation of ATP synthesis during the K^+ uptake showed a competition for energy by the two processes. In the presence of valinomycin and at low extramitochondrial K^+ concentrations, ADP could be rapidly phosphorylated. This occurred with a decrease in net potassium ion uptake. At higher external K^+ concentrations respiratory energy was unavailable for ATP synthesis, and only a portion of added ADP could be phosphorylated within a reasonable time. Further evidence for the competitive nature of the two processes for respiratory energy was from Mg^{++} effects and from timing of valinomycin addition.

Acknowledgements

My most sincere thanks are extended to Dr. Mary Spencer for her leadership, helpful advice and encouragement which was so willingly given.

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LIST OF ABBREVIATIONS

ADP - adenosine 5' diphosphate

ADP/O - adenosine diphosphate to oxygen ratio

ATP - adenosine 5' triphosphate

BSA - bovine serum albumin

DNP - dinitrophenol

EDTA - ethylenediaminetetracetic acid

EGTA - ethyleneglycoltetracetic acid

NADP - nicotinamide adenine dinucleotide phosphate

nmol - nanomoles

RCR - respiratory control ratio

TES - N-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid

Tris - N-tris(hydroxymethyl)-aminomethane

TPP - Thiamine pyrophosphate

INTRODUCTION

With the development of the chemiosmotic hypothesis, an intimate relationship between ion transport and ATP synthesis was seen possible. Although there is much research in these fields, the mechanisms of both ATP synthesis and ion transport have not been completely established.

Density gradient centrifugation offers a method of obtaining mitochondria in a reasonable degree of purity and the use of a zonal rotor offers the advantage of capacity.

Mitochondria isolated from animal tissue have been shown to take up K^+ in an energy-dependent process. Furthermore K^+ is not as susceptible as divalent ions to forming complexes or causing pH changes during uptake processes. Potassium uptake can be monitored by ion selective electrodes. The liquid membrane electrode is a new type of electrode with distinct advantages over that of a glass K^+ specific ion electrode.

Much research has been done on the valinomycin-induced K^+ uptake in animal mitochondria and only recently have such studies been reported for plant mitochondria. The relationship of the valinomycin-induced K^+ uptake and ATP synthesis (as measured directly) has not been done. An investigation was done in this dissertation to examine the characteristics of the two processes in pea cotyledon mitochondria, and to learn more of relationships between the two.

LITERATURE REVIEW

I. Choice of Tissue for Preparation of Mitochondria

Since the present investigation was undertaken to examine certain energy-requiring processes in intact plant mitochondria a suitable tissue source of intact mitochondria was important. Mitochondria which are fully developed and intact can be isolated from cotyledons removed from germinating seedlings. Cotyledon tissue contains a food reserve utilized by the seed during germination. The mobilization of this food reserve is accompanied by a marked increase in cotyledon respiration and energy conservation ability. Isolated mitochondria from such tissues show a close parallel in respiratory activity to that of the whole cotyledon (Öpik, 1965 and Solomos _et_ _al_., 1972).

Respiratory activity of mitochondria is measured by oxygen utilization on a nanomoles (nmol) oxygen utilized per minute per milligram protein basis while intactness may be demonstrated by high respiratory control ratios (RCR) and an adenosine diphosphate/oxygen (ADP/O) ratio approaching theoretical. RCR is the ratio of oxygen utilized per minute in state III respiration (oxygen, substrate, phosphate, and adenosine diphosphate (ADP) not limiting) compared to state IV (ADP limiting) (Chance and Williams, 1955). The ADP/O ratio is the ratio of gram atoms of oxygen utilized by the system during state III respiration with the addition of a given number of μ moles of ADP.

Mitochondria from animal tissue have been much more extensively studied than those from plant tissue. However, Ikuma and Bonner (1967) compared mitochondria from mung bean tissue and rat liver tissue, and showed much higher respiration rates in the plant mitochondria. Mitochondria from various plant tissues show respiratory parameters at least equal to, and in some cases superior to animal mitochondria (e.g. Ikuma and Bonner, 1967; Phillips, 1971).

Öpik (1965) and Malhotra and Spencer (1970) isolated intact mitochondria from bean (Phaseolus vulgaris L.) cotyledons and observed changes in activity when isolated from different ages of cotyledons. Young et al., (1960) and Phillips (1971) showed that mitochondria from pea (Pisum sativum L.) cotyledons reached a maximal activity when isolated from cotyledons five days after imbibition. Biochemical and ultrastructural changes in mitochondria from pea cotyledons during germination have been documented (Solomos et al., 1972).

The demonstration of high respiratory activity, the very high to excellent RCR and ADP/O ratios, and the reproducibility in the isolation of mitochondria at a known stage in development was instrumental in the choosing of pea cotyledonary tissue as a source of mitochondria for the present investigation. In addition, the use of etiolated tissue avoided contamination of the mitochondrial preparation with chloroplasts or chlorphyll.

II. Isolation of Mitochondria from Pea Cotyledons

A. Medium for Tissue Grinding

The components of a good grinding medium usually consist of an osmoticant, a buffer, and other various compounds to remove inhibitory substances released during isolation or to prevent denaturation or oxidation.

The frequently used osmoticants for mitochondrial isolation are mannitol, sucrose, or a combination of both. Sucrose has been observed to have a detrimental effect on mitochondrial integrity (Tedeski, 1965); however, the reason is not clear. Bonner (1967 and 1970) has outlined some of the conditions for isolation of intact mitochondria. Mannitol is of value as an osmoticant since it aids in the separation of starch from mitochondria. Ethylenediaminetetracetate (EDTA) complexes inhibitory heavy metals and Ca^{++}. Calcium ions activate phospholipase which destroys mitochondrial membranes. Ethyleneglycoltetracetate (EGTA) can be employed instead of EDTA to avoid complexing Mg^{++} (an ion which is required for maximum mitochondrial activity). Bonner also suggests using a medium pH near 8 to minimize phospholipase activity although more frequently the pH is around 7.5. Bovine serum albumin (BSA) is used to bind free fatty acids that may otherwise act as uncouplers (Bonner, 1965). Also mitochondria remain in a contracted state in the presence of BSA.

B. Tissue Maceration

Different laboratories have developed their own methods of disrupting tissue and freeing mitochondria from the confines of the cell. Some of the kinds of equipment used include: a Waring Blendor run at low speed, a commercial salad maker, a meat grinder, or a mortar and pestle. Bonner's laboratory highly recommends a soup maker, called Mixer 66, available from Moulinex Company (Bonner, 1967). Storey and Bahr (1969) have used a Polytron, (an attachment that fits a Waring Blendor base) with great success. Previous workers in our laboratory found that a gently used mortar and pestle produced mitochondria of high integrity (Malhotra et al., 1970; Phillips, 1971). It is essential to work quickly and at 0°-4°C to maintain intact mitochondria.

C. Centrifugal Separation of Mitochondria

1) Centrifugal Techniques: In any centrifugation, separation of particles is dependent on two things: size of the particle (a), and density of the particle (ρ). In differential centrifugation, particles are separated basically on their size difference. However, many subcellular particles can be of the same size - hence purity of the fractionated particles is difficult to obtain. With the recognition in the 1950s that density gradient centrifugation allowed particle separation on the basis of density as well as size, a much greater purity of subcellular fractions became possible (Anderson, 1956; de Duve et al., 1959). Thus, density-gradient centrifugation became the method of choice for the separation of biological particles. The major problem with the method was that only a few milligrams of particles could be handled in a conventional "swinging bucket" rotor. Other problems such as "droplet sedimentation effects" and "wall effects" arose that strongly limited the resolution of a zone of larger particles, such as mitochondria (Anderson, 1966).

The development of the zonal rotor for use in density-gradient centrifugation (Anderson, 1966) provided a means for obtaining a large amount of material from a gradient. In addition, greater resolution is possible because the "wall effect" is avoided by means of wedge-shaped sectors in the rotor. With the use of zonal techniques the lysosome contamination of crude mitochondrial preparations (de Duve, 1959) and the peroxisomal contamination of mitochondrial pellets (Plesnicar et al.,

1967) could be minimized. Although peroxisomes are nearly the same size and density as mitochondria, the peroxisome is freely permeable to sucrose while the mitochondrion is not. The bound water of the peroxisome exchanges for sucrose; thus the peroxisome increases greatly in specific density with time, and will ultimately move to a density of 1.25-1.26 in sucrose. Mitochondria, meanwhile, retain their bound water and move to a sucrose concentration similar to their original specific density (1.201 as reported by Solomos et al., 1972). Thus mitochondria and peroxisomes are separated in sucrose density gradients on the basis of differing osmotic properties of their membranes.

2) Gradient Materials: Sucrose has been commonly used as a gradient material for mitochondria, although sorbitol affords better preservation of structure (Avers et al., 1969). Other gradient material includes dextrans (glucose polymers) or Ficoll, a sucrose polymer. Although solutions of these two polymers are not very dense, equilibrium density occurs in corresponding less dense solutions since membrane organelles do not lose water when exposed to them. Also the organelles may be more stable during isolation (Laties and Treffry, 1969). Cesium chloride is sometimes employed when a very high density is required, but its cost for routine work is otherwise prohibitive. Lately some workers have used silicone oil gradients for isolation of intact mitochondria (Harris and Van Dam, 1968). For the present zonal work, sucrose appeared to be the satisfactory choice.

3) Isopycnic Separations: For the first ten years after the introduction of density gradients f[illegible]cellular isolations (Anderson, 1956), investigators simply employed linear gradients.

However, linear gradients are optimal only for isopycnic centrifugation. The use of step gradients, convex or concave gradients offers advantages under certain circumstances. For example, capacity is greatly increased for isolation of plant mitochondria when a step gradient is used. Solomos et al., (1972) prepared pea cotyledon mitochondria in a swinging bucket rotor by loading a crude preparation of mitochondria over 38% w/w, 44.8% w/w, 48% w/w and 60% w/w bands and centrifuging it for 2½-3 hours at 149,000 g in a L2-65B Spinco ultracentrifuge. Although few workers have employed step gradients for isolation of mitochondria in zonal rotors, this type of gradient is preferred when a large amount of mitochondria is required because of the large capacity of the gradient.

III. Oxidative Phosphorylation in Plant Mitochondria

A. History

The complexity of oxidative phosphorylation and the difficulties in attempts to explain its mechanism prompt a reiterance of Racker's rather memorable quote, "Anyone who is not confused about oxidative phosphorylation just doesn't understand the situation."

The basic concepts of oxidative phosphorylation owe their origin to many of this century's early biochemists but it wasn't until the late 1930s that phosphorylation was linked to the respiratory chain. The first papers of Kalackar, Belitzer and Ochoa demonstrating this are translated and presented in Kalackar's recent book (Kalackar, 1969). Ion transport was thought to be quite separate from oxidative phosphorylation until the development of the chemiosmotic hypothesis showed an intimate relationship was possible. Through all of this period three basic hypotheses were developed for oxidative phosphorylation - each with its own proponents.

It was through the development of the chemiosmotic and conformational coupling hypotheses that many new lines of investigation were opened. Today most workers in the field accept the hypothesis that the electron transport chain is involved in a proton pump (Lehninger, 1972). However, it is unclear whether this proton pumping is the direct result of the arrangement of the electron transport chain components or secondary to the chemical or conformational coupling processes. Also, it remains to be shown whether the proton gradient is the direct driving force for ATP synthesis. As a result, the most active field of research in mitochondrial function during the last few years concerns problems related to membrane conformation, compartmentation, and ion translocation.

B. Theories of Oxidative Phosphorylation

1) Chemical Coupling: The historical aspects of ATP formation in enzymatically catalyzed reactions immediately led to a suggestion for an intermediate of a chemical nature to enable the coupling of electron transport to ATP synthesis (Slater, 1958). By the beginning of the 1960s, isolation of the high energy intermediate seemed imminent. Nevertheless, the isolation of the chemical intermediate, and therefore proof of the chemical coupling theory, did not materialize.

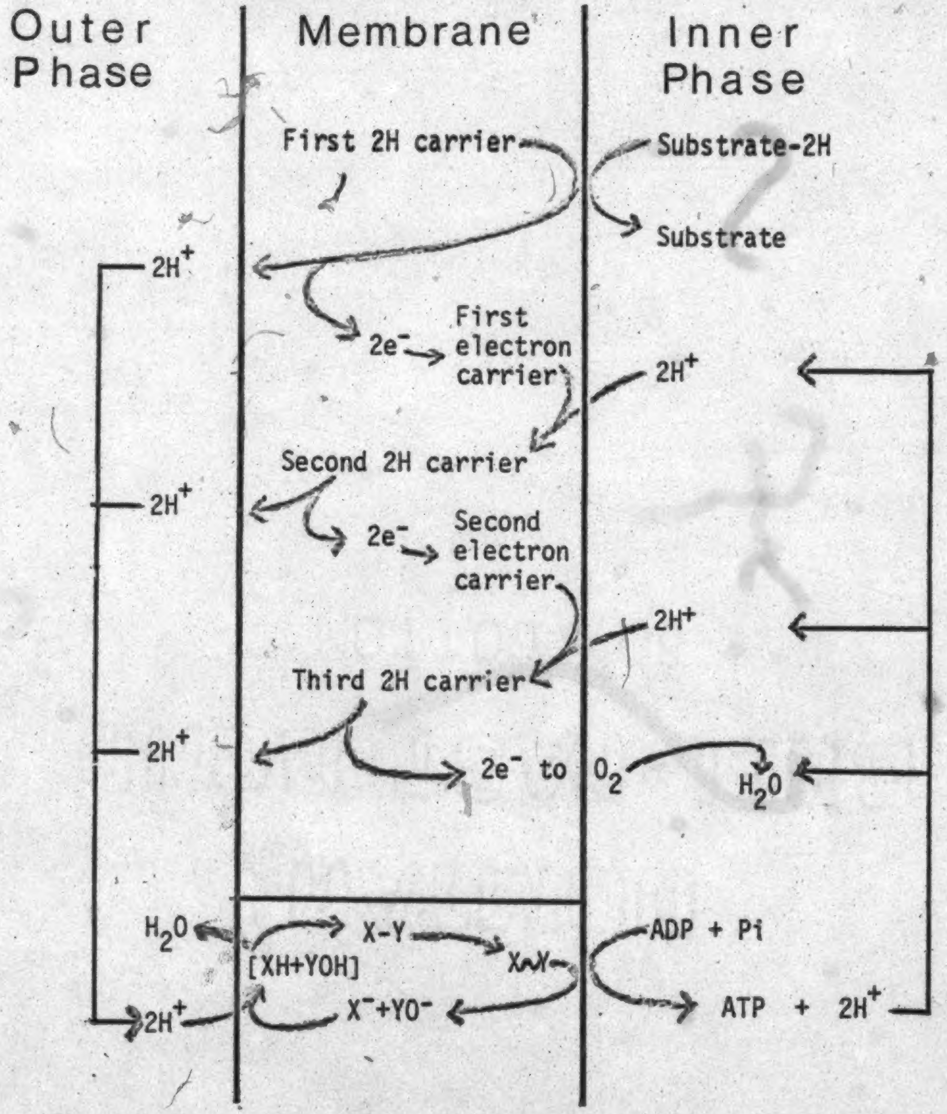
2) Chemiosmotic Coupling: The first proposal for a chemiosmotic type of mechanism linking electron transport and ATP synthesis came in 1961 (Mitchell, 1961). Later this was revised somewhat and detailed to a greater extent (Mitchell, 1966 and 1969). Although

largely ignored by biochemists at first because of the biophysical nature of the hypothesis, the demonstration that a pH gradient could drive ATP synthesis in chloroplasts (Jagendorf and Hind, 1963) gave great support to the hypothesis. Mitchell rejects the chemical high energy intermediate and substitutes for it instead, a pH gradient and/or a membrane potential that are created by the separation of protons and electrons during respiration.

During respiration the electron transfer chain is thought to develop a "proton motive" force across the membrane by depositing the protons (removed from the substrate) on the outer side of the membrane. This is thought to occur by the following steps. The substrate is considered to donate two hydrogens to the first hydrogen carrier of the chain (See Fig. 1). The first acceptor reduces the second carrier of the electron transport chain, which requires only electrons for reduction. The protons, which are not required for this reduction, are separated from the electrons. As a result of the spatial arrangement of the first carrier in the chain, the protons are deposited on the outer side of the membrane. As the next carrier is reduced its requirement for both protons and electrons is met by oxidation of the second carrier and removal of protons from the inner phase. With the requirement of the third carrier for only electrons for reduction, separation of H^+ and electrons again occurs with the protons deposited on the outer phase. This cycle repeats still one more time. In total then, 6 H^+ are deposited on the outer phase of the membrane as shown in the scheme in Fig. 1. The driving force for ATP synthesis comes from the lower concentration of H^+ thus produced in the inner phase, and the

Fig. 1. Chemiosmotic hypothesis. The build-up of hydrogen ions on the outer phase of the mitochondrial membrane (as a result of the nature and orientation of electron transport chain components) drives the production of the high energy intermediate (X~Y). The intermediate in turn drives ATP synthesis with $2H^+$ deposited in the inner phase for every molecule of ATP formed.

CHEMIOSMOTIC SCHEME OF OXIDATIVE PHOSPHORYLATION


Outer Phase
Membrane
Inner Phase
First 2H carrier
Substrate-2H
Substrate
2H+
2e-
First electron carrier
2H+
Second 2H carrier
2H+
2e-
Second electron carrier
2H+
Third 2H carrier
2H+
2e- to O2
H2O
H2O
X-Y
[XH+YOH]
X~Y
ADP + Pi
2H+
X-+YO-
ATP + 2H+

(from Racker, 1970)

higher concentration of H^+ on the outer phase of the membrane. The details of how this is accomplished presents the biggest problem. It is visualized that 2 H^+ from the outer phase go to H_2O through loss of water when XH and YOH react to form $XY + H_2O$. XY becomes the high energy intermediate as it moves toward the inner phase of the membrane and reacts with ADP and Pi to form $ATP + YO^- + 2H^+ + X^-$. The compounds, X^- and YO^-, return to the outer side of the membrane, and again form XH and YOH through the uptake of two more protons. Thus the original pH gradient formed during electron transport is dissipated with one ATP molecule formed for every two protons that move back to the inner phase. Since 6 H^+ moved to the outer phase during electron transport, three ATP molecules can be formed for every two electrons transported.

Although the scheme is controversial it provides sound rationale for many of the observations of oxidative phosphorylation and the requirements of the coupling process. The strongest objections come from lack of a consistent stoichiometry of ion transport data and from exchange reaction experiments. Nonetheless, the present state of knowledge seems to point most favourably in the direction of the chemiosmotic hypothesis.

3) Conformational Coupling: Although some investigators tend to regard the conformational coupling theory as a special case of the chemical coupling hypothesis, most feel it is sufficiently different to be regarded as a separate hypothesis. Raaflaub (1954) was one of the originators of the conformation coupling hypothesis with his work on swelling and contraction changes in mitochondria induced by different respiratory substrates, ATP, and Mg^{++}. Boyer (1965) developed more

detailed concepts of the hypothesis. Conformational coupling refers to a mechanism in which electron transfer to or from an electron carrier protein induces an energy-rich conformational state in the protein, which is somehow transmitted to the ATP-forming enzyme. Presumably the free energy for the formation of the high-energy phosphate bond comes from the breaking of several non-covalent bonds of low energy. Some researchers now feel that the final solution to the mechanism of oxidative phosphorylation will come from a compromise proposal arising from the three coupling theories (Racker, 1970; Lehninger, 1972).

IV. Characteristics of Plant Mitochondria

A. Oxidation and Phosphorylation

Following isolation, mitochondria from plant tissue can be shown to oxidize NADH, malate, succinate, isocitrate, and α-oxoglutarate (Bonner, 1967); and often at a rate higher than those obtained with animal mitochondria. The inclusion of thiamine pyrophosphate, (TPP) is necessary, however, for oxidation of α-oxoglutarate in mitochondria from etiolated seedlings.

The rate of these oxidations is greatly affected by the presence of ADP. When ADP is not limiting the resultant respiration is termed "state 3," as opposed to the ADP-limited "state 4." As described earlier, the respiratory control ratio (RCR), which is the $\frac{\text{state 3}}{\text{state 4}}$ ratio, indicates the extents of mitochondrial intactness and coupling of oxidation to phosphorylation. The RCR in intact mitochondria, (see Ikuma and Bonner, 1967), for succinate as substrate is generally 3-5, with malate (4-6), NADH (3-4) and much greater (8-15) with α-oxoglutarate as substrate (see Phillips, 1971).

The ADP/O ratios for different substrates utilized by plant mitochondria are similar to those of animal mitochondria (Ikuma and Bonner, 1967). The ratio should be very close to 2 for succinate, 3 for malate or NADH and 4 for α-oxoglutarate. When the mitochondria are uncoupled or loosely coupled, however, the ADP/O ratio is greatly reduced as a result of a faster turnover of ATP (Racker, 1970).

B. Other Energy-Linked Functions of Plant Mitochondria

Besides the production of ATP in mitochondria, there are a number of other energy-linked functions in mitochondria - namely, ion transport, reversed electron transport, transhydrogenation and swelling and contraction. Most of the research in these areas has been carried out on animal mitochondria.

As in any biological area, the attempt to relate structure to function is always paramount; however, the biophysical nature of energy-linked functions poses problems of structure-function relationships that we are just now beginning to understand.

C. Mitochondrial Membranes

1) Similarity to Membrane Models: Many synthetic membranes have been investigated with the objective of reproducing the biological system (Huang et al., 1964; Mueller et al., 1964; and Henn, 1970). Until quite recently the membrane model, advanced by Davson and Danielli in 1935, has been regarded as the best representation of how biological membranes look. This model, in essence, is based on a bimolecular lamella of phospholipid - with hydrocarbon chains constituting

the middle of the membrane, and the hydrophilic portion of the phospholipid bounded by protein.

Various researchers, such as Green and co-workers (1966) have come to dispute this hypothesis. Green reasons that a subunit structure must exist in mitochondria. This would be necessary to accommodate his observations that: mitochondrial membrane proteins bind hydrophobically to phospholipids in a precise stoichiometry, and two, membranes can be fragmented by detergents into homogeneous lipoprotein complexes. Both Davson's and Danielli's, and Green's models have certain merits. With recent experimentation on membrane models, Danielli's model has survived surprisingly well. (See review by Thompson and Henn, 1970).

In comparing the physical properties of a biological membrane and an artificial lipid bilayer model, striking similarities can be seen. This adds support for the bilayer model for biological membranes (Thompson and Henn, 1970). The greatest discrepancy between the actual and the model membranes occurs in measurements of electrical resistance. The lipid bilayer model shows a resistance of 3 to 5 orders of magnitude larger than naturally occurring biological membranes. Also, the permeabilities for the alkali metals in bilayer models are all about the same. In contrast, in biological membranes, there are very marked differences in peremeabilities to different cations. A most interesting phenomenon results when the electrical resistance of these artificial membranes is lowered by the presence in the bilayer of methanol, a substance with a relatively high dielectric constant. Ion selectivity can result (Miyamoto and Thompson, 1967). An examination of dinitrophenol (DNP) effects on model lipid bilayers shows that DNP can lower the electrical resistance of the structure by an amount that would be sufficient to account for the uncoupling in mitochondria. (Bielawski, Thompson and Lehninger, 1966). Other uncouplers have been found to have the same effect on bilayers. Furthermore, the decrease in resistance results exclusively from increased H^+ and/or OH^- conduction (Hopfer et al., 1968). Miyamoto and Thompson (1967) observed that Fe^{+++} at 10^{-5}M lowers the electrical resistance of a lecithin tetradecane bilayer by a factor of 10^3. Furthermore the bilayer showed anion selectivity. Cations such as Cd^{++}, Mn^{++}, and Cu^{++} increase the electrical resistance of phosphatidyl choline membranes. Results for model membranes similar to the very high discrimination among cations by biological membranes have not yet been reported, except for experiments employing a number of macrocyclic peptides on bilayer membranes. Mueller and Rudin (1967) observed that valinomycin, a cyclic polypeptide, produced a larger increase in permeability of K^+ than Na^+ in model membranes. Andreoli et al. (1967) showed that there is a drop in resistance of 5 orders of magnitude in a model lipid membrane, and that the selectivity for K^+ was 200-300 X larger than Na^+ in the model. Also the order of permeability of other alkali metals was the same as that observed in most biological membranes, i.e.

$$P_{H^+} > P_{Rb^+} > P_{K^+} > P_{Cs^+} > P_{Na^+} > P_{Li^+}$$

Valinomycin, meanwhile, has been shown to uncouple oxidative phosphorylation and stimulate respiration in animal mitochondria (Pressman, 1965). These effects are associated with an uptake of K^+ and extrusion of H^+.

Although model membranes can only be regarded as an approximation of biological membranes; their use in developing structure-function relationships should not be underestimated.

2) Structure of Mitochondrial Membranes: With the development of the electron microscope it became possible to study the structures of mitochondrial membranes in great detail. Unexpectedly scientists found that mitochondria contained an inner membrane in addition to the outer bounding membrane.

Various research efforts have identified particular pathways and enzymatic activities with different parts of the mitochondria. Clearly the components of the respiratory chain and oxidative phosphorylation are bound to the inner membrane and must be spatially arranged in ordered arrays.

The work of Racker and collaborators (See Review Racker, 1970) have identified a protein molecule with high adenosine triphosphatase (ATPase) activity and found it to be associated with the inner membrane.

The fine structure of mitochondrial membranes is far from being understood. The fact that nearly all the lipid can be extracted from the inner membrane and yet the remaining protein maintains the "unit membrane" structure is indicative of the important role protein plays in membrane structure (Fleischer et al., 1967).

In order to account for the observed properties of the mitochondrial inner membrane the Danielli model has to be refined somewhat. Assuming the lipid bilayer is the structural backbone and the general permeability barrier, there must be local modifications or special sites on the membrane to account for observed permeabilities. Various proteins are bound to both sides of the lipid surfaces in ordered arrays such that they can carry out highly specialized functions.

The outer membrane of mitochondria can be shown to have very different properties than that of the inner membrane in animal mitochondria at least, where most studies have been done. The outer membrane is apparently permeable to substances both charged and uncharged, with molecular weights up to 10,000. The inner membrane has very restricted permeability except to small molecular weight uncharged molecules. (Klingenberg and Pfaff, 1966; Werkheiser and Bartley, 1957).

The outer membrane bursts in hypotonic solutions rather than unfolding as the inner membrane does. This principle has been employed in separating the two membranes (Parsons, et al., 1966). The most distinguishing feature of the two membranes chemically lies in their lipid composition. The outer membrane in animal mitochondria contains about 45% phospholipid by dry weight, or two to three times more phospholipid than the inner membrane (Parsons and Yano, 1967) with the balance mainly protein. It is quite apparent that the inner membrane, in which resides most of the enzyme machinery for substrate oxidation, respiration and energy conservation, is most important in the present study.

V. Ion Transport

A. Ion Transport Antibiotics

The fact that certain polypeptides and other compounds are capable of changing the electrical resistance of biological membranes has already been pointed out. Although the resultant cation selectivity of the membrane is probably related somehow to decreased membrane resistance for certain uncouplers, the mechanism of action of ionophores* such as valinomycin, nigericin and gramicidin is probably different (Läuger, 1972).

It has been suggested that the peptide ring of valinomycin acts as a hydrophobic cage for a particular ion, and thus permits it to pass through the hydrocarbon portion of the bilayer (Mueller and Rudin, 1967; Läuger, 1972). In this way valinomycin acts as an enzyme; that is, it reduces the extremely high energy barrier that must be surmounted for the transport of an alkali ion across the hydrophobic interior of a lipid membrane. Although the affinity of K^+ for the central cavity of valinomycin is rather low, valinomycin is an effective carrier because of its high turnover number (Läuger, 1972). A single molecule of valinomycin is capable of transporting 10^4 K^+ ions per second.

Although it is interesting to speculate that actual biological ion carriers may be of a chemical nature similar to valinomycin and enable passage of specific ions through the membrane; this still does not explain the mechanism of active transport. The increase, in permeability of a membrane to an ion does not explain accumulation of that ion. At least some of the other ionophores probably act in the same way as valinomycin. Monactin and enniactin B share a common property with valinomycin; they are macrocyclic molecules in which one side of the ring is hydrophilic, the other hydrophobic. Also, they are capable of forming complexes with alkali ions to a high degree of specificity.

B. Ion Transport as Viewed by the Chemiosmotic Hypothesis

During electron transport, hydrogen ions are transported to the outer phase of the membrane so that a proton gradient is formed. The membrane prevents the protons from moving back into the inner phase except in stoichiometric exchange via carriers for a cation (termed "antiport" transport), or accompanied by an anion ("symport"), electrical neutrality thus being maintained. If these latter processes were to occur very quickly the chemical gradient of the protons may be nearly dissipated; but the total membrane potential as a result of the gradients of other ions remains high. Now if an anion is introduced on the negative side and a cation on the positive side of the membrane and each moves to the opposite side (termed "uniport" transport) a dissipation of the membrane potential results. With the loss of membrane potential, more protons can now be released through operation of electron transport. In such a case the H^+ gradient tends to be maximized, while the membrane potential component is minimized. Together, the proton gradient component and the membrane potential, provide the "high energy intermediate" of ATP synthesis. Mitchell suggests that the high-energy intermediate of mitochondria is almost entirely an electrical potential with the pH component being only minimal. Support for this arises from work of Addanhi and Sotos (1969) and Lardy and Ferguson (1969), which indicates that there is a minimal pH gradient across the mitochondrial membrane although the system readily phosphorylates ADP. Thus the original H^+ gradient must be transformed to electrical potential presumably by a rapid antiport transport of Pi or other anions (Palmieri _et al_., 1970, Papa _et al_., 1969), or possibly by a Na^+/H^+ symport exchange (Mitchell and Moyle, 1969).

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*Ionophore is a term introduced by Pressman which refers to a special class of antibiotics that facilitate transport of specific ions across membranes.

As already discussed, if valinomycin is added to a mitochondria suspension in the presence of respiratory substrate; K^+ moves into the mitochondria via an electrically neutral complex. The amount of K^+ transported is very small, unless one of the following applies: an anion can move with K^+, a cation moves out of the mitochondrion, or an opposite electrical potential is somehow applied.

Although some researchers (see Henderson, 1971) think that net cation transport occurs only under these rules, Pressman (1970) feels that valinomycin acts by increasing the supply of K^+ to an energy-linked K^+ pump. It is possible that operation of a K^+-ATPase can act as such a pump.

C. Ion Transport in Mitochondria

1) General Ion Transport: Work on ion transport in plant mitochondria has been carried out largely by Hanson and co-workers (e.g. Truelove and Hanson, 1966) and Yoshida et al. (1968, a, b). Some of the more intensive investigations on animal mitochondria were done by Chappell (e.g. Chappell and Haarhoff, 1967), Lehninger (e.g. 1970), and Pressman (e.g. Harris et al., 1966). The relationship between ion transport and energy conservation appears to be an intimate one, although the biophysical nature of it makes it complex.

Although various types of ion transport in mitochondria were extensively investigated in the 1950s, ion transport by mitochondria was regarded mostly as an interesting curiosity and was not thought to be related to electron transport because of the lack of a consistent stoichiometry. Then in 1961, Vasington and Murphy discovered Ca^{++} could be accumulated in large amounts by respiring liver and kidney mitochondria. This was considered rather amazing since Ca^{++} had previously been shown to inhibit phosphorylation (Lehninger, 1949). With Mitchell's presentation of the chemiosmiotic hypothesis and with the demonstration that protons could be ejected during the energized uptake of Ca^{++}, Mg^{++}, Mn^{++}, and K^{+}, (see review Pressman, 1970) it became apparent that ion transport might be intimately related to electron transport through the proton pump. Recent reviews on the current status of ion transport include those of Henderson (1971), Lehninger _et al_. (1967), Harris, Judah and Ahmed (1972), and Moore (1971; also 1972).

The inner membrane of the mitochondrion is believed to be the site of specific translocators for various charged molecules that cannot readily diffuse through this membrane. Among the anion translocators described by Chappell (1968), are those for phosphate, malate, succinate, α-oxoglutarate, citrate, isocitrate, glutamate, and aspartate. In addition, Klingenberg and Pfaff (1968) demonstrated an atractylate sensitive ATP-ADP carrier system. The inner membrane is also the binding site for various divalent cations (Thomas and Greenawalt, 1968), which are transported in an energy facilitated process. It is also the site for different ionophores that facilitate transfer of univalent cations (Lehninger, Carafoli, and Rossi, 1967).

2) Potassium Ion Transport: Potassium is an element that is required to a great extent by growing plants. An external concentration of potassium of the order of 10^{-5}M is usually sufficient to support normal growth, but only because the plant's roots have the ability to accumulate potassium in concentrations from 100 to 1,000 times those in its environment (Price, 1970). Some thirty or so enzymes require

activation by alkali ions. In most cases K^+ is the most powerful activator. Pyruvate kinase for example attains maximum activity in the presence of 50 mM K^+, which is approximately the internal cellular concentration of K^+ (Evans and Sorger, 1966).

Pressman and Lardy (1952) were the first to report that animal mitochondria contained a high concentration of K^+. Later, Stanbury and Mudge (1953) showed that mitochondria were able to accumulate K^+ ion in the presence of oxygen and substrate. Furthermore the K^+ uptake could be abolished by low levels of DNP. After a period of stagnation, work on ion transport became revitalized with Mitchell's proposal of the chemiosmotic hypothesis.

Christie and co-workers (1965) showed that animal mitochondria depleted of K^+ could accumulate K^+ against a concentration gradient in the presence of ATP and $MgCl_2$. The discovery of antibiotic-mediated ion transport in mitochondria was made by Moore and Pressman (1964). Valinomycin was shown to induce an energy dependent uptake of K^+ in isolated mitochondria. In subsequent work (Cockrell et al., 1966; Harris et al., 1966), the K^+ movements were monitored by the use of specific ion electrodes. Changes in sodium ion concentration, pH, O_2 utilization and mitochondrial volume were monitored simultaneously. Potassium ion uptake was detected in the presence of reduced nicotinamide adenine dinucleotide (NADH) but efflux occurred with the addition of rotenone. When ATP was added in the presence of rotenone, K^+ uptake was again sustained. Oligomycin could not block the uptake of K^+ when NADH provided the energy, but did block the ATP-induced uptake. Thus it appeared that ATP sustained K^+ transport through an energy transfer to the high energy intermediate.

When K^+ is taken up by rat liver mitochondria, fluorescence data indicates the mitochondria begin to swell (Harris et al., 1966). Recently, Wilson et al. (1972) have examined the effects of addition of valinomycin to plant mitochondria on respiration and swelling. Valinomycin facilitates the energy-dependent uptake of K^+ with a concommitant increase in rate of respiration and swelling. When respiration is terminated and the gradient lost, osmotic contraction follows.

The energy required for the influx pumping of K^+, as induced by valinomycin, has been determined by a number of workers (Pressman, 1965; Cockrell et al., 1966; Rossi and Azzone, 1969) in animal mitochondria and recently by Kirk and Hanson (1973) in plant mitochondria. Values for the number of K^+ transported per high energy bond utilized ($\sim$) have differed from 3.2 to 7.9 in animal mitochondria; and from .58 to .97 $K^+/\sim$ in corn mitochondria. This lack of consistency of stoichiometry for ion transport argues against the chemiosmotic hypothesis. However, changes in other energy-dependent reactions could affect the stoichiometry as could the degree of coupling.

D. ATPase and Mitochondrial Ion Transport

When mitochondria accumulate ions against a concentration gradient, an expenditure of energy is required. This energy could be supplied either by ATP or directly through the electron transport chain. Lately, various groups have attempted to link ion transport with ATPase activity (a non-specific term that refers to an enzyme that hydrolyzes ATP.)

Since cotyledonary tissue provides the nutrients for the actively growing seedling, Lai and Thompson (1972) proposed the idea that energy for transport of these nutrients from the cotyledon would be obtained by hydrolysis of ATP. They found that both basal and Na^+-K^+-stimulated ATPase were present in bean cotyledon tissue and increased in activity up to day two after imbibition followed by a gradual decrease to day 10. Phillips (1971) noted that ATPase activity in pea cotyledon mitochondria increased to a maximum five days after imbibition, the same time that mitochondria show the highest respiratory activity. These results are consistent with the expected role of cotyledon ATPase in supplying energy for the transport of nutrients to other parts of the seedling.

Lehninger (1972) suggests that membrane ATPases might have evolved from cell membrane transport ATPases whose original function was to use ATP to transport nutrients or ions into the cell. Later the cell learned how to reverse the process as well - that is, how to pump ions to make ATP. This idea might explain why the mitochondrion preferentially uses energy to pick up Ca^{++}, rather than phosphorylating ADP when both are available (Rossi and Lehninger, 1964).

Wenner and Hackney (1969) showed that an uptake of Na^+ and K^+ in mouse liver mitochondria correlated with ATPase activity. The possibility is suggested that addition of an ionophore makes Na^+ and K^+ available on the membrane to activate the ATPase, which otherwise is protected from the ionic environment by the membrane. Phillips (1971) also found a stimulation of mitochondrial ATPase with an ionophore, valinomycin. It was shown to stimulate the K^+-ATPase and the Na^++ K^+-ATPase but not the Mg^{++}-ATPase activity.

Cordes and workers (Jain et al., 1969) added ATP and a purified

Na^+-K^+ ATPase preparation (from synaptic vesicles of the rat brain) to a two compartment system separated by a black lipid membrane. An active transport of Na^+ and/or K^+ occurred across the membrane, in proportion to the concentration of ATP. Furthermore ouabain, which inhibits ATPase activity, decreased the Na^+ and K^+ transport.

Cockrell and workers (1967), and Rossi and Azzone (1970), have shown that dissipation of a potassium gradient can be utilized to make ATP. This might be through reversal of a K^+-stimulated ATPase, although later work indicates that an uptake of H^+ occurs, and it is this that drives ATP synthesis (Cockrell, 1972). Thus there is at least some evidence that ion transport is related to ATPase activity. In such a case ion transport in mitochondria probably affects, or may even exert some control, on respiratory activity.

MATERIALS AND METHODS

I. Growth Conditions and Plant Material

Various types of plant tissue have been used as a source of mitochondria. It was decided to use etiolated cotyledonary tissue of pea (*Pisum sativum* L. var. Homesteader) as a source of mitochondria for this work. It has had the advantages of being readily available, reproducibly grown in a short time, and may be easily harvested at a relatively uniform age. The structural development and physiological activity of mitochondria from this tissue is known from work of Solomos *et al.*, (1972) and Phillips (1971). These workers were able to obtain from etiolated pea cotyledons mitochondrial preparations with a longer than normal *in vitro* life and showed no loss in respiratory chain components.

A supply of the untreated seed was obtained from a local seedhouse (Seed Centre Ltd., Edmonton) and was stored in a cold room prior to use. Sufficient seeds to yield 300 g fresh weight cotyledons were placed in water and allowed to imbibe water. After soaking four hours, the seeds were planted in peat flats in horticultural grade vermiculite. Germination was at 26° in the dark. Daily watering was handled by the greenhouse staff. The tissue was called one-day old 24 hours after planting.

II. Isolation of Mitochondria

Cotyledons were picked from five-day old tissue since they yield mitochondria of maximum respiratory parameters (Phillips, 1971) and were harvested just prior to isolation of mitochondria. Seedcoats, hypocotyls and epicotyls were removed and the cotyledons washed in tap water, rinsed with distilled water and kept cold prior to maceration.

The grinding medium was of the following composition: 0.5 M Mannitol, 2 mM EDTA, 0.5% w/v BSA (b grade), and 50 mM N-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid (TES) with buffer pH adjusted to 7.4 at 0° with N-tris(hydroxymethyl)-aminomethane (Tris).

One hundred and fifty grams of cotyledons were ground in 300 ml grind medium for each isolation. By use of an ice-cold mortar and pestle and chilled "grind medium", temperature throughout the grinding remained between 0 - 3°C. Extreme care was taken during maceration to prevent damaging of the mitochondria. Best results were obtained by allowing the pestle to gently fall from 1½ - 2 inches above the bottom of the mortar at the rate of 120 strokes per minute. Grinding time was eight minutes. The brei was strained through one layer of miracloth previously rinsed with distilled water.

The resulting filtrate (about 250 ml) was quickly edge-loaded onto a Ti-XIV zonal rotor spinning at 1500 rpm at 2° C in a Beckman Spinco L2-65B centrifuge. A sucrose step gradient followed, with steps made up of 50 ml of 20% w/w sucrose, 50 ml of 30% w/w sucrose, 50 ml 35% w/w sucrose, 100 ml of 37.5% w/w sucrose and sufficient 41.5% w/w sucrose to completely load the rotor. In order to maintain high values for respiratory parameters it was found necessary to include .1% BSA in all the sucrose steps, as well as including sufficient TES (made to pH 7.4 at 25° with Tris) to bring the pH of the sucrose solution to 7.2 - 7.4.

After the rotor was loaded at 1500 rpm the rotor was speeded up to 5000 rpm, capped and taken to speed. Centrifugation was for one hour at 37,000 rpm. After deceleration the rotor was then edge-unloaded at 1500 rpm by use of 50% w/w piston sucrose. An LKB Uvicord (type 4701A), connected to a Beckman 10 mv potentiometric recorder, was used to monitor protein peaks in the elutant. The protein peak corresponding to the fraction from the interface of the 37.5% and 41.5% steps was saved for subsequent work. Electron microscopy and measurement of enzymatic activities indicated this was the mitochondrial fraction.

The fraction was diluted with cold distilled water and rapid stirring to an approximate 20% sucrose concentration. The sample was centrifuged at 20,000 x g for 10 minutes and the resulting pellet resuspended in suspend buffer. The suspend buffer was of the following composition: 0.3 M mannitol, 1.0 mM EDTA, and 25 mM TES, with the pH adjusted to 7.2 at 25° with Tris. In order to give a concentration of 10 mg mitochondrial protein per ml, 3 ml of suspend buffer was used. When comparisons of this zonal mitochondrial preparation with a crude mitochondrial preparation were made, Phillips'(1971) method of isolation of the crude preparation was used. Most of the chemicals used in the present work were of the highest purity obtainable and were purchased primarily from Fisher Scientific. Special chemicals that were purchased from Calbiochem included BSA, Antimycin a, and valinomycin. Rotenone, TES, Tris, oligomycin, NADP and the hexokinase, glucose 6-P dehydrogenase enzyme mixture were purchased from Sigma.

III. Measurement of Mitochondrial Respiratory Parameters

Mitochondria isolated by the above procedure were tested for the respiratory control ratio (RCR), ADP/O value and oxygen utilization.

These values were measured at 25° with a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) equipped with a Clarke oxygen probe. The apparatus was connected to a Beckman 100 mv potentiometric recorder with a chart speed of 0.5 in. per minute to provide a continuous tracing. The assay was made in 3.2 ml of assay medium with the following concentrations: 0.3 M mannitol, 4 mM $MgCl_2$, 5 mM KH_2PO_4, sufficient Tris to adjust the pH to 7.2 at 25°. In addition one of the following substrates was included: 8 mM succinate, 8 mM α-oxoglutarate (with 5 mM malonate and 0.07 mM thiamine pyrophosphate (TPP), or 8 mM malic (with 0.07 mM TPP).

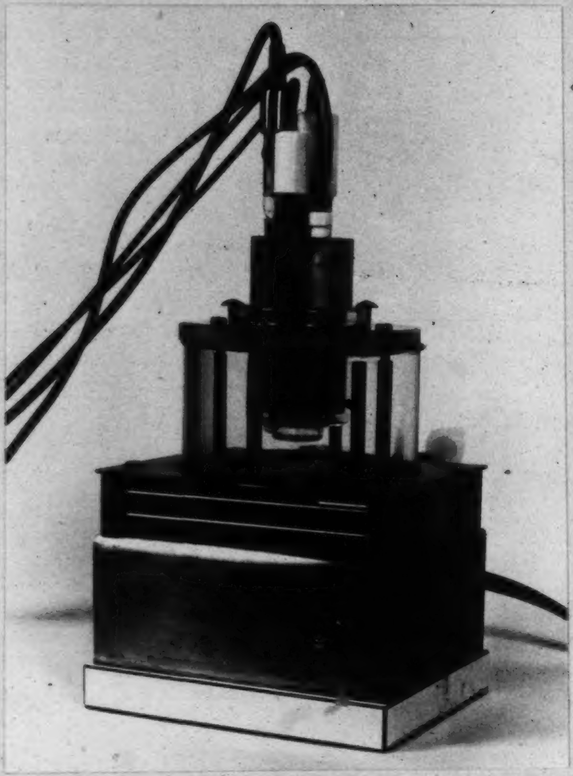
RCR values were determined from the ratio of the oxygen utilization in state III over the oxygen utilization in state IV. ADP/O values were obtained by calculating the nanoatoms of O consumed, sustained by the addition of 250 μmoles ADP. The concentration of oxygen in air-saturated assay medium was assumed to be 225 μM at 25° following calculations and testing reported by Stinson (1968) and Phillips (1971).

The activity of catalase was assayed polarographically by use of a slightly modified version of the method of Breidenbach _et al_. (1968). To 4 ml of 0.1 M phosphate buffer, pH 7.0 which had been previously freed of oxygen by boiling followed by cooling under nitrogen, 5 μmoles of H_2O_2 were added. To this mixture, 50 μl of enzyme solution were added. The rate of oxygen evolution (in μmoles O_2 formed per milligram protein per minute) was calculated from the initial linear portion of the recorder tracing.

IV. Measurement of Ion Uptake

The early work of Pressman (1967) provided the basis for design of equipment employing ion selective electrodes for monitoring ion activity measurements. A YSI water bath assembly was modified so that a custom built glass vessel of 1.75 inches inside diameter would fit snugly into the top of the bath (Fig. 2). The vessel was designed to include a shallow well for a circular magnetic stirrer and another well to accommodate the lower part of the bulb of the pH electrode. A plexiglass probe assembly was also custom built to hold the various electrodes and when fully inserted into the glass vessel, provided space for an assay mixture volume of 10 ml ± 2%. The probe assembly provided for the simultaneous measurement of K^+, Na^+, pH and O_2 concentration when the appropriate electrodes were used. The K^+ movements were monitored with an Orion Model 92-19 liquid-membrane potassium electrode connected to a Radiometer Model 26 Expanded Scale pH Meter. Although the electrode's principle of operation is based on valinomycin; no interaction between the valinomycin of the electrode and that in the test system occurred. Output of the pH meter was recorded on a single channel on a Texas Servo Riter dual pen recorder. Equipment electronics necessitated the incorporation into the circuitry of a 100 mv turnpot (built by the Technical Services Department of the University). (See appendix re: Calculation of K^+ uptake.) Oxygen concentration was monitored by a Clarke oxygen probe connected to a YSI Biological Oxygen Monitor, and recorded on the other channel of the dual pen recorder. For numerous reasons as discussed later, the Na^+ movements were not routinely monitored. Solution pH was routinely monitored with an

Fig. 2. Apparatus for measurement of potassium, sodium, pH and oxygen. Closeup of probe assembly shows: a) K^+ electrode; b) combination pH electrode; c) Na^+ glass electrode; d) Clarke oxygen probe; e) glass reaction vessel and f) modified YSI waterbath assembly.



e
f

A. H. Thomas L 15 4858 Combination pH Electrode connected to a Radiometer Model 28 pH meter with pH changes recorded on a Beckman 10 mv potentiometric recorder. (See appendix re: Electrodes). The reference element of the pH probe was used as the common reference for the other two electrodes.

The use of ion selective electrodes provided a rapid, sensitive, and continuous monitoring of changes in ion activity.

V. Estimation of ATP

A fluorometric assay for the estimation of ATP was carried out after termination of mitochondrial reactions with perchloric acid. This was followed by extraction and assay of ATP fluorometrically following the method of Estabrook _et al_. (1967). The technique permits multiple sampling during a single experiment as well as multiple assays on each sample.

Synthesis of ATP was studied in a reaction vessel at 25°C with mitochondrial protein concentrations approximately 1 mg per ml. At specified times, an aliquot of the reaction mixture (usually 1.0 ml) was removed from the reaction vessel and pipetted to 2 ml of 7% perchloric acid in a conical centrifuge tube. After a waiting period of 10 minutes in ice, the samples were centrifuged at 1200 x g for ten minutes. An aliquot of 2.0 ml was carefully removed and pipetted to a small beaker. The samples were neutralized with constant stirring to a pH of 7.0-7.2 with a neutralizing buffer with the following concentrations: 0.5 M triethanolamine, (adjusted to pH 7.4 with HCl), and 0.67 N KOH. Approximately 1.8 ml of buffer was required for neutralization of sample. Following a 15 minute storage period in ice, the samples were centrifuged to remove potassium perchlorate. The clear supernatant was stored in the cold until used for the assay of ATP. An aliquot (usually 0.3 ml) was removed and added to a cuvette containing 4 ml of buffer of the following composition: 50 mM triethanolamine, (adjusted to pH 7.4 with HCl), 10 mM KCl, 10 mM Mg_2Cl_2, 100 μmoles NADP, 10 μmoles D-glucose. The cuvette was placed in a Turner Model III Fluorometer, and the background fluorescence compensated for through the instrument circuitry. A small volume (representing 1 unit enzymatic activity*) of a hexokinase, glucose 6-phosphate dehydrogenase (2:1) enzyme mixture was added to start the reaction. The increase in fluorescence was monitored on a Beckman 10 mv potentiometric recorder and compared to the fluorescence on addition of 5.0 nmoles of ATP from a standard solution. The filters used transmitted at 366 mμ and <420 mμ.

VI. Determination of Protein

The Lowry method for the quantitative determination of protein, (Lowry et al., 1951) as modified by Phillips (1971), was used to determine mitochondrial protein concentration. Mitochondrial protein was first precipitated in each sample by addition of 0.1 ml 20% TCA per 0.1 ml of the sample. After a 10 min storage in the cold, the samples were centrifuged at 1200 x g for five minutes. The supernatant layer was discarded and the pellet dissolved in 0.5 ml 1 N NaOH. The volume was made to 1.0 ml with distilled water. Five ml of a mixture that was 0.02% $CuSO_4$, 0.04% in potassium tartarate and 4% Na_2CO_3

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*One unit will oxidize 1.0 μmole of glucose-6-PO_4 to 6-Phosphogluconate per min at pH 7.4, 25°C, in the presence of NADP.

(in 0.1 N NaOH) was added, and the mixture incubated for fifteen minutes at 25°C. Subsequently 3.5 ml water and 0.5 ml of a 1.0 N phenol-water mixture (1:1 v/v) was added to each tube. Following a thirty minute incubation at room temperature the optical density of the solution was measured at 750 nm on a Beckman DU spectrophotometer. Human serum albumin was used as the standard.

RESULTS

I. Preparation of Mitochondria by Zonal Ultracentrifugation Techniques

A. Isolation of a Zonal Fraction

In the present investigations a relatively large amount of mitochondrial protein of high purity was required. Since zonal techniques can be employed to satisfy these criteria, an investigation into the most suitable conditions for a zonal isolation of mitochondria was carried out. As it is important to work quickly when isolating mitochondria the possibility of avoiding any time-consuming steps was also investigated.

In preliminary investigations, use of a continuous gradient did not prove satisfactory for isolation of a mitochondrial band with high resolution*. Since a step gradient is the method of choice for isolation of particles as large as mitochondria and chloroplasts, (M. Griffith, personal communication), a step gradient was employed.

A graph (Fig. 3) of transmittance versus volume of elutant showed good resolution in a run where 100 ml crude homogenate was center-loaded on 50 ml 20%, 50 ml 30%, 50 ml 35% and 100 ml 37.5% followed by 200 ml 41.5% and 100 ml 48% w/w sucrose bands. In order to isolate a greater quantity of mitochondrial protein, however, an increased size in homogenate volume was investigated. It was found that a sample of crude homogenate as large as 250 ml could be edge-loaded onto the rotor followed by a step gradient with carefully selected volumes for each step such that good resolution of the mitochondrial fraction was obtained. The sample was loaded onto the rotor in a matter of only a few minutes by employing the centrifugal force of the rotor as a pump. By edge-loading the sample, starch granules present in the homogenate sedimented rather quickly onto the walls of the rotor. Disruption of the gradient (that could have occurred when the sample was center-loaded onto the rotor) was thus avoided. An overlay solution was found unnecessary with the large sample. Centrifugation at 37,000 rpm for one hour provided a good separation of mitochondria from other cellular debris. Following centrifugation the rotor was unloaded through the center line and the elutant monitored for peaks of low transmittance. A photographic reduction of a tracing of the volume of elutant versus transmittance is shown in Fig. 4. A strong absorbance peak showed in the region of the interface of the 37.5% and 41.5% w/w sucrose bands. This was considered to be the mitochondrial band in view of work of Solomos et al. (1972).

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*Resolution refers to the difference in distance between the centers of two particle zones divided by the sum of the standard deviations. It is greatly dependent on the width of particle zones (Price, 1970).

B. Examination of the Zonal Fraction

An electron micrograph of organelles present in this band is shown in Fig. 5. Little contamination by sub-cellular organelles other than mitochondria is present. Also most of the mitochondria show fully developed membranes.

In further tests of this peak (Table I) the mitochondrial band displayed good respiratory parameters in comparison to the values for the high density fraction that emerged from the zonal ultracentrifuge after the mitochondrial fraction, and the values for a crude mitochondrial

Fig. 3. Tracing of volume of elutant and transmittance. One hundred ml of sample was center-loaded on a Ti-XIV zonal rotor with 50 ml 20%, 50 ml 30%, 50 ml 35%, 100 ml 37.5%, 200 ml 41.5% and the balance 48% w/w sucrose. A 50 ml overlay solution followed the sample. Centrifugation was at 37,000 g for 1 hr.

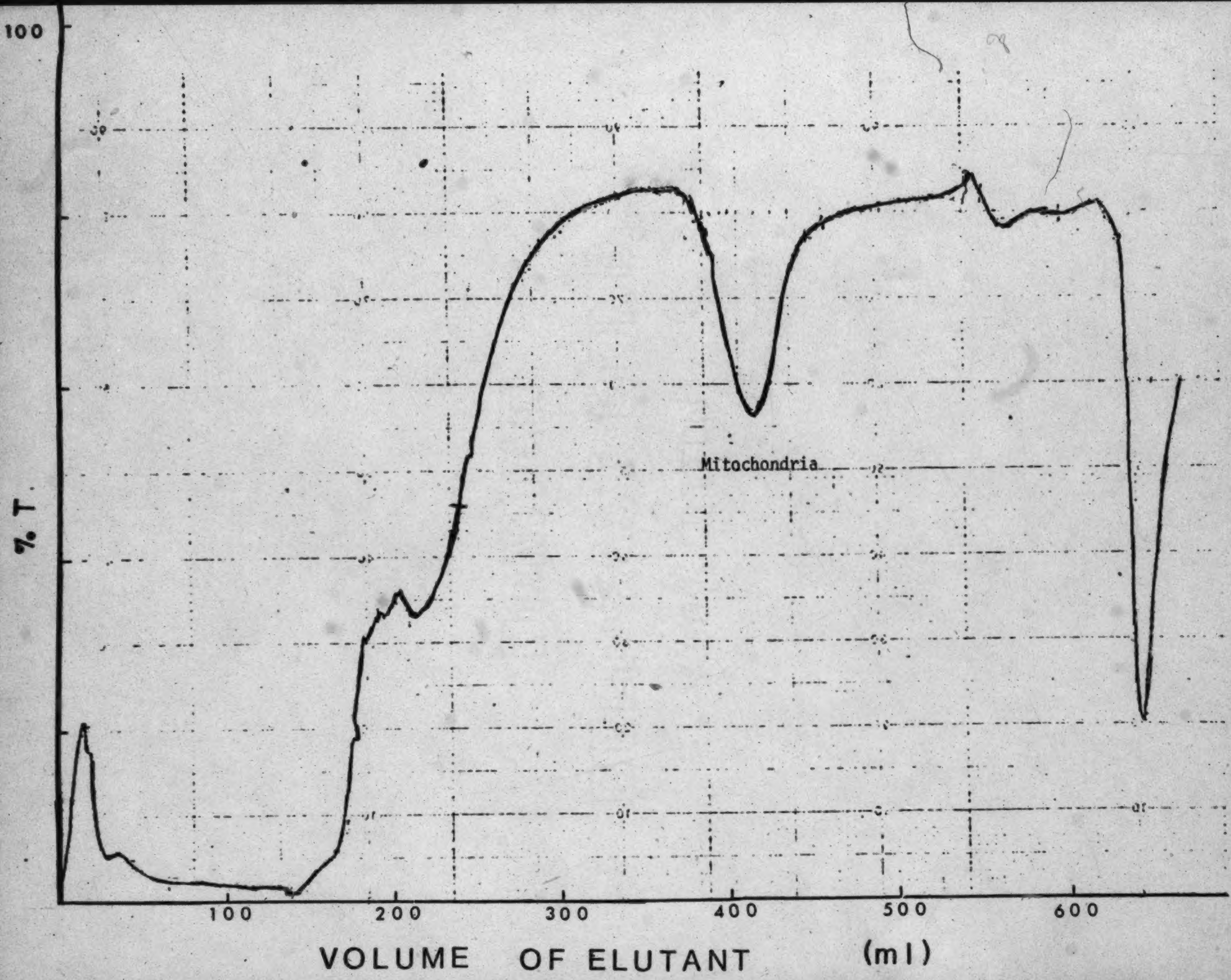
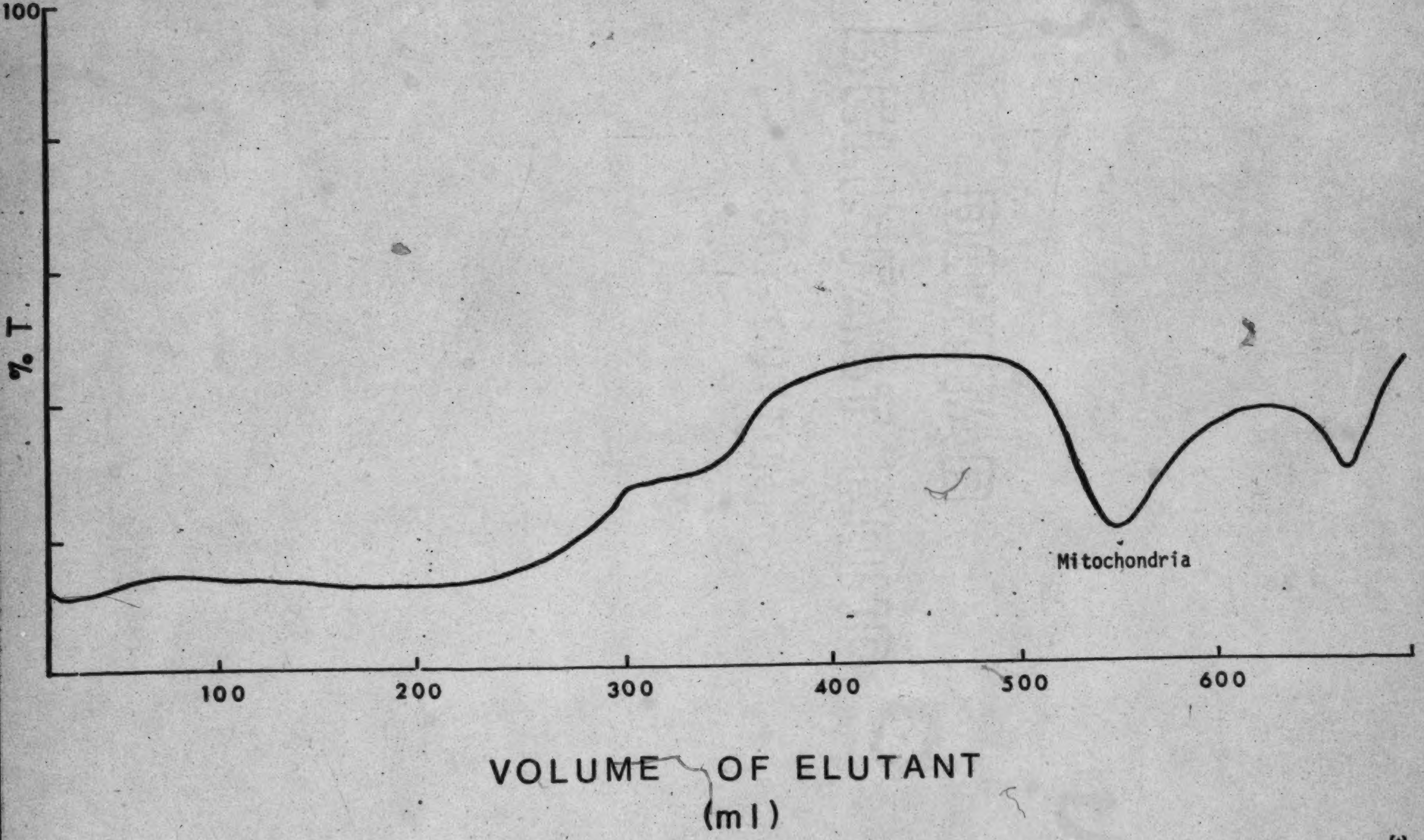

100
% T
Mitochondria
100
200
300
400
500
600
VOLUME OF ELUTANT (ml)

Fig. 4. Tracing of volume of elutant and % transmittance. A sample of 250 ml of pea cotyledon homogenate was edge-loaded into a Ti-XIV zonal rotor followed by 50 ml 20%, 50 ml 30%, 50 ml 35%, 100 ml 37.5% and the balance, 41.5% w/w sucrose. Centrifugation was at 37,000 *g* for 1 hr.


100
% T
Mitochondria
100
200
300
400
500
600
VOLUME OF ELUTANT
(ml)

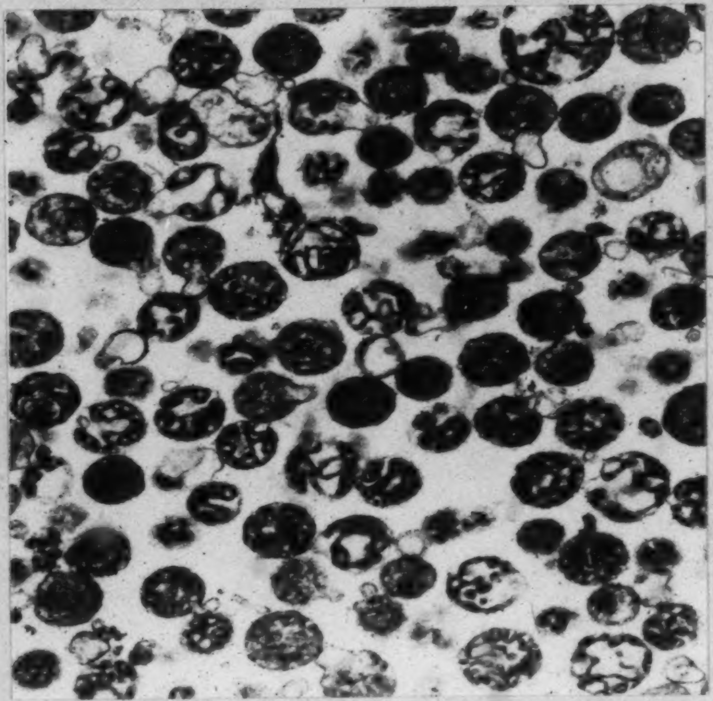


Fig. 5. Electron micrograph of mitochondrial fraction isolated in Ti-XIV zonal rotor. Mitochondria were fixed in 3% glutaraldehyde 3-4 hours and post-fixed for 2 hours in 2% (OsO_4). Thin sections were stained in uranyl acetate followed by lead citrate. Results show mainly mitochondria present, 23,000 X magnification. Mitochondrion (m).

TABLE I. Properties of a Zonal Mitochondrial Fraction, a High Density Fraction, and a Crude Mitochondrial Preparation

	Respiratory Parameters			
	RCR	ADP/O	O_2 Utilization	Catalase Activity
			n mol O_2 utilized/min/mg protein	n mol O_2 evolved/mg protein/min
Zonal Mitochondrial Fraction	7.3	3.1	177	35
Zonal High Density Fraction	1.0	1.0	31	845
Crude Mitochondrial Preparation	4.8	3.0	78	310

All fractions were isolated from etiolated cotyledons five days after imbibition*. Assay was done at 25°C on a YSI Biological Oxygen monitor using 3 ml of buffer of the following composition: 0.3 mM mannitol, 25 mM TES, 4 mM $MgCl_2$, 5 mM KH_2PO_4 and a pH of 7.2 at 25°C adjusted with Tris. Substrate was α-oxoglutarate at a concentration of 8 mM. The crude 'mitochondria' were isolated by centrifugation at 40,000 g for 5 min of the supernatant layer (obtained from the initial spin at 2,500 g for 7 min).

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*Similar results were obtained in similar preparations from cotyledons 4 and 6 days after imbibition.

preparation.

In addition the low activity of catalase ($H_2O_2:H_2O_2$ oxidoreductase, EC 1.11.1.6) present in the zonal mitochondrial band in comparison with the other samples was further evidence for lack of peroxisomal contamination.

II. Study of Potassium Ion Movements in Mitochondria

A. Characteristics of the System

1. Potassium Ion Movements and Oxygen Concentration

The equipment and assembly used for the study of K^+ transport in pea cotyledonary mitochondria has been previously described (See Materials and Methods Section).

Following equilibration of the potassium electrode with 10 ml of assay buffer solution, usually 0.5 mM or 1.0 mM in KCl, mitochondrial suspension was added through a narrow port in the probe assembly wall. Since the electrode senses the extramitochondrial* K^+ concentration, an increase in extramitochondrial K^+ concentration would reflect a net K^+ efflux from the mitochondria. Alternatively, a decrease in extramitochondrial K^+ reflects a net K^+ uptake by the mitochondria. However if the electrode indicated a very rapid change in extramitochondrial K^+ concentration (a matter of a few seconds or less), it was considered that transport processes were not responsible for the concentration change. When a sample from a crude preparation of mitochondria was added the electrode very quickly indicated an increase in extramitochondrial K^+. It was thus assumed that the K^+ concentration of the mitochondrial suspension was higher than that of the assay buffer; thus causing an increase in extramitochondrial K^+. When a sample of a zonal mitochondrial preparation was used, no immediate deflection of the electrode was apparent; hence the K^+ concentration in the suspension must have been very near, or less than, that of the assay buffer.

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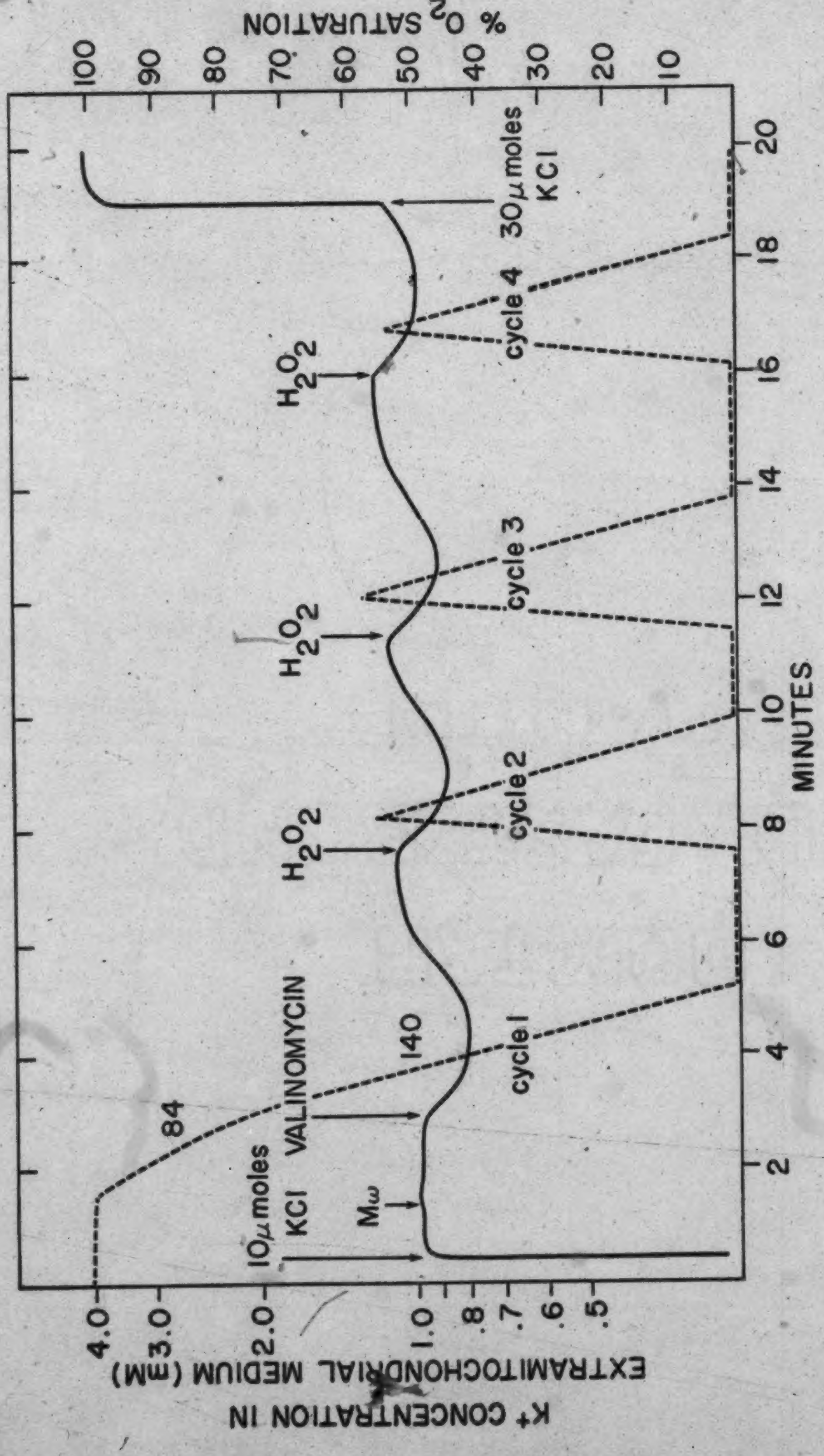
*Extramitochondrial refers to the solution that the mitochondria are suspended in.

Following addition of mitochondria, (M_ω) a small and gradual change in extramitochondrial K^+ was often observed. In some samples a small increase in extramitochondrial K^+ was detected, while in others a small decrease in extramitochondrial K^+ occurred while on addition of some preparations, no change in extramitochondrial K^+ occurred. This inconsistency was likely related to the internal concentratión of K^+ in the mitochondria.

When the potassium electrode reached a stable potential in the presence of mitochondria, valinomycin was added (0.5 μg per ml suspension) to initiate the induced K^+ movements (see Fig. 6). Almost immediately the electrode began to show a linear decrease in extramitochondrial K^+ (indicating uptake). The trace gradually tailed off until a stable potential was observed. The total time required for a steady trace was usually of 1½ - 2 minutes after addition of valinomycin (see Figure 6). (If no mitochondria were present, addition of valinomycin did not cause the electrode to respond hence interaction between valinomycin in the electrode and added valinomycin did not occur.)

The rate of oxygen utilization can also be seen to greatly increase at the point of valinomycin addition. It was consistently found that valinomycin caused approximately a doubling in oxygen utilization when the extramitochondrial K^+ concentration was 0.5 or 1.0 mM. After a

Fig. 6. Dual pen recorder tracing of potassium ion movements and oxygen concentration. (—) represents potassium ion concentration and (--) shows respiration for a mitochondrial preparation suspended in a buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10.0 mM sodium acetate, 5.0 mM sodium phosphate and 1 mM KCl with the buffer pH adjusted to 7.2 at 25°C with Tris. Potassium movements were initiated by the addition of 0.5μg valinomycin/ml suspension. Mitochondrial protein concentration was 14 mg/10 ml buffer. Solution was made aerobic with addition of 5 μmoles H_2O_2.


% O_2 SATURATION
100
90
80
70
60
50
40
30
20
10
K+ CONCENTRATION IN EXTRAMITOCHONDRIAL MEDIUM (mM)
4.0
3.0
2.0
1.0
.8
.7
.6
.5
MINUTES
2
4
6
8
10
12
14
16
18
20
10μ moles KCl
Mω
VALINOMYCIN
84
140
cycle 1
H_2O_2
cycle 2
H_2O_2
cycle 3
H_2O_2
cycle 4
30μ moles KCl

number of minutes and at the point when the oxygen concentration in the solution approached 0%, an efflux of potassium was detected.

In the present work the efflux of K^+ gave rise to an extramitochondrial K^+ concentration equal to the K^+ concentration prior to uptake. The number of nanoequivalents (nequiv) of K^+ required for the observed change in extramitochondrial K^+ concentration was calculated and the result expressed as the 'total' K^+ taken up or effluxed as the case may be. Since it was desireable to know if K^+ could again be taken up under aerobic conditions it was decided to employ the catalase contamination of the mitochondrial preparation (see Table 1) to make the solution aerobic. Figure 6 demonstrates that when H_2O_2 was added, rapid conversion to O_2 apparently took place and the suspension became aerobic. At the same time the mitochondria began to take up K^+ to the same extent, and at approximately the same rate, as that of the original uptake.

It can also be noted that the same thing is repeated throughout a third cycle and also into a fourth, although the magnitude of the movements began to decrease. One further observation from Fig. 6 is that upon addition of 30 μmoles of KCl the electrode responded very rapidly with a steady trace after a few seconds. This demonstrated that electrode and instrument response is not a limiting factor in tracing K^+ movements for the levels of KCl used experimentally.

2. Reproducibility Among Cycles of the Same Sample

Even with zonal techniques, the amount of mitochondrial protein obtained in a single run was a limiting factor in experiments on K^+ transport. The possibility was investigated of using the cycles of

TABLE II. Reproducibility of Potassium Ion Movements Among Cycles in One Mitochondrial Preparation

Cycle	'Total' K^+ Uptake nequiv/mg protein	Initial Rate of K^+ Uptake nequiv/mg protein/min	'Total' Efflux of K^+ nequiv/mg protein	Initial Rate of K^+ Efflux nequiv/mg protein/min
1	290	540	300	410
2	290	680	290	380
3	290	710	280	390
4	250	610	240	360

Results were obtained from a zonal preparation of mitochondria from 5 day old etiolated cotyledons. The assay buffer was composed of 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate, 5 mM sodium phosphate and 1.0 mM KCl. The buffer was adjusted to pH 7.2 at 25°C with Tris. 'Total' K^+ refers to the net loss or gain of K^+ from the extramitochondrial phase during the appropriate phase of the cycle. Mitochondrial protein concentration was 9.3 mg/10 ml buffer. K^+ movements were initiated by addition of 0.5 μg valinomycin/ml of buffer. The assays were done at 25°C.

uptake and efflux as a tool whereby different compounds could be added to test their effects on K^+ uptake in subsequent cycles.

The data in Table II show that the total amount of K^+ taken up on a nequiv per mg protein basis is the same for each of the first three cycles but has decreased slightly by the fourth. Cycle 1 refers to the original uptake induced by valinomycin while cycles 2, 3, and 4 refer to uptake in the transition from anaerobic to aerobic conditions with each consecutive addition of H_2O_2. The rates are not entirely reproducible; however, this is probably caused by error in determination of initial rate from the small linear portion of the tracing of each movement. 'Total' efflux of K^+ was always very nearly equal to that of the 'total' uptake, as one might expect. However, the rate of efflux, which reflected the rate of dissipation of the gradient was substantially less than the rate of uptake.

Thus, the reproducibility of total uptake and efflux throughout the first three cycles is demonstrated. Because of experimental error, only very large differences in rates were considered to be significant.

3. Concentration of Valinomycin and Induced Potassium Ion Movements

The concentration of valinomycin necessary to initiate a maximum uptake of K^+ was investigated. Valinomycin present at very low levels was able to sustain maximum ion uptake (Table III). Although 'total' uptake of K^+ reached a maximum at 0.25 μg valinomycin per ml mitochondrial suspension the rate did not reach a maximum until the valinomycin concentration was 0.5 μg/ml.. In almost all subsequent work valinomycin was added to give a concentration of 0.5 μg/ml mitochondrial suspension.

TABLE III. Effects of Concentration of Valinomycin on Potassium Ion Movements

Concentration of Valinomycin	'Total' Uptake	Initial Rate of Uptake	'Total' Efflux	Initial Rate of Efflux
μg/ml mitochondrial suspension	nequiv/mg protein	nequiv/mg/min	nequiv/mg protein	nequiv/mg protein/min
0.05	34	80	34	85
0.25	85	170	85	85
0.5	85	255	85	102
1.0	85	255	85	102

Results are reported for mitochondria from the same isolation from etiolated cotyledons 5 days after imbibition. Reaction conditions are as in Table II except KCl concentration was 0.5 mM. Valinomycin was dissolved in 95% ethanol such that 10λ contained 0.5 μg valinomycin. Protein concentration was 5.1 mg protein/10 ml buffer.

4. Reproducibility Among Samples from the Same and Different Preparations

Table IV compares rates and total movements of K^+ in duplicate samples of the same preparation. The data indicate that fairly reproducible values are obtained for 'total uptake' between samples. Similar results were found in other preparations. It was noted however that fairly large differences could occur for 'total' uptake and rates among samples from different runs.

B. Respiratory Parameters and Potassium Ion Movements

Since a greater permeability could be inferred to cause a lower net uptake an investigation of intactness of mitochondria and their ability to take up K^+ was done. Since the possibility exists that potassium ions may simply absorb onto the mitochondrial membranes with valinomycin treatment, it was desirable to determine if the observed K^+ uptake could take place in a suspension of mitochondria that had lost their intactness and were thus highly permeable to K^+. The potassium uptake ability was thus investigated in a suspension of uncoupled mitochondria and a suspension of tightly coupled mitochondria.

Table V shows that when mitochondria of poor respiratory parameters were isolated, they lacked ability to accumulate K^+ to any measureable extent. Such mitochondria were isolated simply by employing a harsh tissue grinding technique during the initial steps of preparation. However, when a mild grinding technique was used, mitochondria demonstrating good respiratory parameters were isolated and exhibited an ability to accumulate K^+ to a measureable extent. Thus it would appear

TABLE IV. Reproducibility of K^+ Uptake Among Samples From the Same and Different Preparations

Mitochondrial Preparation	# Sample	Total K^+ Uptake nequiv/mg protein	Rate of K^+ Uptake nequiv/mg protein/min
1	I	290	460
	II	290	540
2	I	210	390
	II	220	420

Typical results of potassium uptake values for mitochondria isolated from 5 day etiolated cotyledons. The assay mixture was of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate, 5 mM sodium phosphate, and 1.0 mM KCl. The buffer was adjusted to pH 7.2 with Tris. Valinomycin was added to give a concentration of 0.5 µg/ml mitochondrial suspension. Protein concentration was 10.0 mg/10 ml buffer. Assays were done at 25°C.

TABLE V. Comparison of Potassium Ion Transport in Mitochondria of Differing Respiratory Parameters

	Respiratory Parameters			K^+ Movements	
K^+ Conc	RCR	ADP/O	O_2 UTILIZATION nmoles/min/mg protein	'TOTAL' nequiv/mg protein	RATE nequiv/mg protein/min
0.5 mM	1.0	0	107	0	0
0.5 mM	2.4	2.1	130	110	300
0.5 mM	3.0	1.8	160	140	280
1.0 mM	1.0	0	80	0	0
1.0 mM	2.5	1.9	115	240	530
1.0 mM	3.3	1.9	120	210	470

Results are reported for mitochondria isolated with different grinding techniques from etiolated cotyledons five days after imbibition. Assay was done at 25°C in a reaction buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate, and 5 mM sodium phosphate. pH of the buffer was adjusted to 7.2 at 25°C with Tris. Potassium movements were initiated with addition of 0.5 µg valinomycin/ml suspension. Respiratory parameters were assayed separately in a buffer of the following composition: 0.3 M mannitol, 8 mM succinate, 4 mM $MgCl_2$, 5 mM potassium phosphate and 50 mM TES. pH was adjusted to 7.2 with Tris.

that mitochondria with a high degree of intactness are necessary for K^+ accumulation.

Although values for the RCR in intact mitochondria should be of the order of 3 - 5 when succinate is used as substrate (Bonner, 1970), mitochondria were used in experiments on K^+ transport only if they exhibited a RCR of 2.5 or better in the second cycle of ADP addition. Only slight differences in K^+ transport can be seen for mitochondria with a RCR of 2.5 as compared with 3.0 or higher.

C. Sodium Ion and Hydrogen Ion Changes During Potassium Movements

As described in a previous section (Materials and Methods), an O_2 probe, a glass pH electrode for pH measurements and a glass electrode for measurement of Na^+ were all incorporated into the design of the probe assembly so that attendant changes in these variables could be monitored. The dependency of K^+ uptake on the presence of O_2 has already been discussed. However under the experimental conditions employed no changes in pH or sodium ion concentration were observed. The sodium ion electrode was highly erratic and slow in response. In addition sodium salts of acetate and phosphate were used in the assay buffer hence the extramitochondrial sodium concentration was too high to detect any changes in concentration of Na^+ of the order of magnitude of the K^+ changes. Although no pH changes as a result of K^+ movements were evident, the pH was routinely monitored throughout most of the runs since changes in H^+ could affect the response of the K^+ electrode, albeit to a limited extent.

D. Effects of Different Respiratory Substrates on Potassium Ion Movements

The requirement for a respiratory substrate (Table VI) as well as oxygen was further proof that the K^+ uptake was energy dependent. When no substrate was present, no observable K^+ uptake occurred. Succinate has been observed by previous workers in our laboratory (see Phillips, 1971) to facilitate a faster O_2 utilization than either malate or α-oxoglutarate in respiring mitochondria under State III conditions. This observation was also apparent in the results reported here and is opposite to that reported by Wiskich and Bonner (1963) in sweet potato mitochondria. In addition to the higher O_2 utilization, succinate also sustained somewhat larger 'total' uptake of K^+ and the fastest rate. Since succinate sustained the best K^+ movements and fastest O_2 utilization under conditions reported here, it was decided to routinely employ succinate as substrate in further investigations of K^+ transport.

E. Requirement of Anions for Potassium Ion Uptake

Harris et al. (1966) have reported that inorganic phosphate is necessary to achieve high rates of K^+ uptake in mitochondria from rat liver tissue.

A K^+ concentration of 0.5 mM was employed in the present work (Table VII) to observe the effects of anions on K^+ uptake since greater accuracy in measurement of small changes in K^+ concentration could be achieved than at higher extramitochondrial K^+, even though lower 'total' uptake and slower rates are apparent for the former. Chloride supplied

TABLE VI. Effect of Different Respiratory Substrates on Potassium Ion Uptake

Substrate	K^+ Uptake		Respiratory Parameters		
	Total	Rate		O_2 Utilization nmol/mg protein/min	
	nequiv/mg protein	nequiv/mg protein/min	RCR	O_2 Units	ADP/O
1. None	0	0	-	-	-
2. Succinate 20 mM	210	620	3.0	94	1.7
3. Malate 20 mM	160	525	3.0	66	1.9
4. α-Oxoglutarate 20 mM	130	270	3.3	36	3.3

Results* are reported for mitochondria from the same preparation isolated from etiolated pea cotyledons six days after imbibition. Each assay was done with 9.7 mg mitochondrial protein at 25 in 10 mls of a buffer of the following composition: 0.3 M mannitol, 10 mM sodium acetate, 5 mM sodium phosphate, 1.0 mM KCl plus one of the substrates indicated. In addition thiamine pyrophosphate (70 μM) was present in both the malate and α-oxoglutarate buffers. The buffers were adjusted to pH 7.2 at 25 C with unneautralized Tris. Potassium uptake was initiated by addition of 0.5 μg of valinomycin/ml buffer. Values for respiratory parameters were obtained from an average of the second and third cycles of ADP addition (0.25 μmol/cycle). Buffers for these measurements of respiratory parameters included 4 mM $MgCl_2$.

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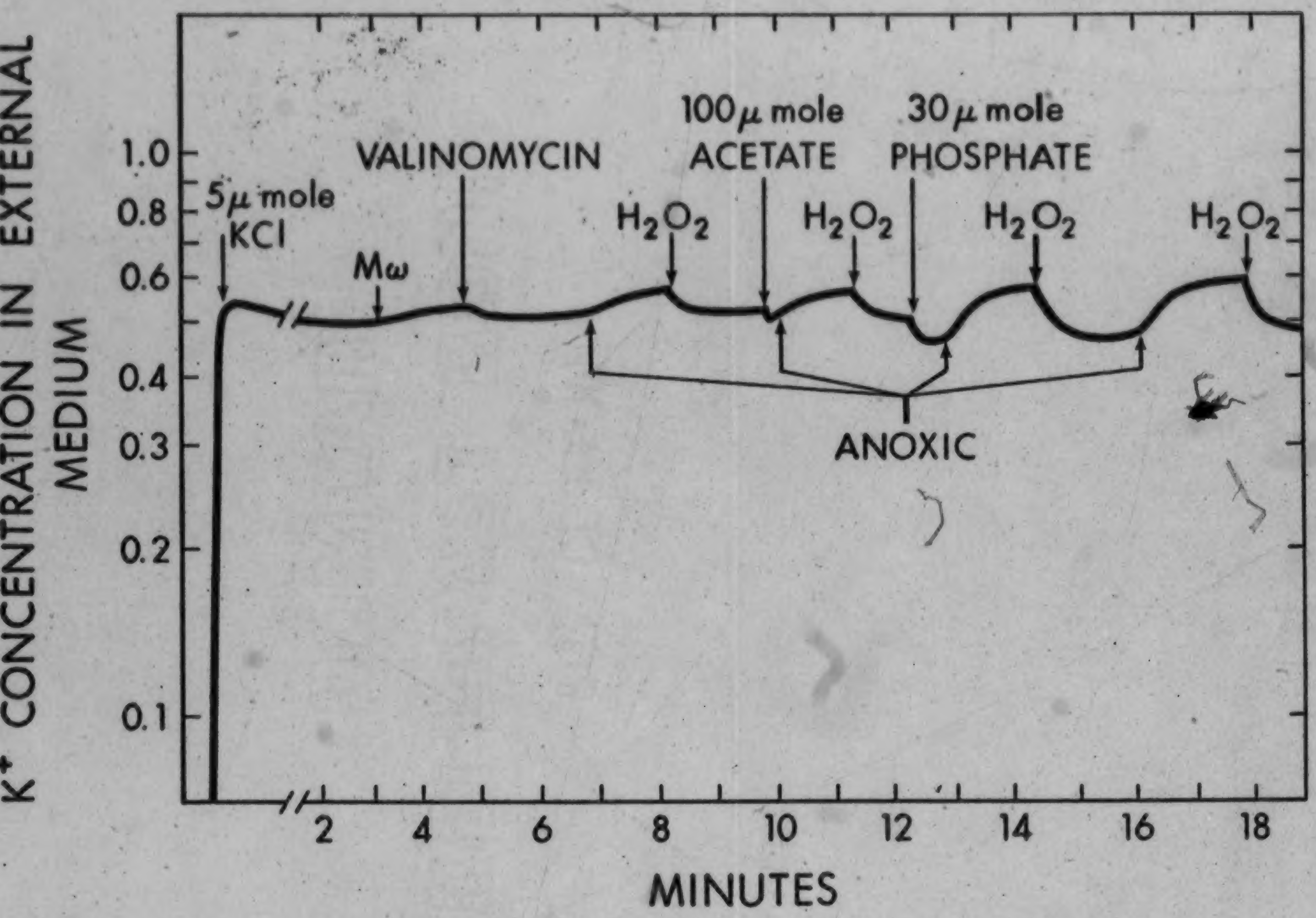
*Similar results were noted in other runs.

TABLE VII. Requirement of Anions for Potassium Ion Transport

	K$^+$ Uptake			
	RUN I		RUN II	
Salt(s) added	'Total' K$^+$ nequiv/mg protein	Rate	'Total' K$^+$ nequiv/mg protein	Rate
1. Control	30	slow	30	slow
2. 5mM Sodium Chloride	30	slow		
3. 5mM Sodium Acetate	41	slow	47	slow
4. 2.5mM Sodium Phosphate	91	fast	85	fast
5. 5mM Sodium Phosphate	110	fast		
6. 5mM Sodium Phosphate + 5mM Na Acetate	120	fast	135	fast
7. 5mM Sodium Phosphate + 10mM Na Acetate	140	fast	160	fast
8. 10mM Sodium Phosphate + 10mM Sodium Acetate	120	fast		

Results are reported for mitochondria isolated from etiolated pea cotyledons six days after imbition. Assay was done at 25°C in a reaction buffer consisting of 0.3M Mannitol, 20mM Succinate and the anions in concentrations as given above. pH was adjusted to 7.2 at 25°C with Tris. Potassium movements were initiated by addition of 0.5 µg valinomycin/ml suspension. External K$^+$ concentration was 0.5 mM. Respiratory parameters indicated that the mitochondria were intact. Rates are expressed qualitatively since the rates for the first three samples were difficult to accurately determine quantitatively. Protein concentration was 6.6 mg/10 ml buffer.

Fig. 7. Effects of anions on potassium ion movements. Mitochondria were isolated from etiolated cotyledons five days after imbibition. Assay was done in a buffer with the same composition as in Table VII. Potassium movements were initiated by addition of 0.5 μg valinomycin/ml suspension. Protein concentration was 6.9 mg/10 ml buffer. Respiratory parameters indicated that the mitochondria were intact.


K+ CONCENTRATION IN EXTERNAL MEDIUM
1.0
0.8
0.6
0.4
0.3
0.2
0.1
MINUTES
2
4
6
8
10
12
14
16
18
5μ mole KCl
Mω
VALINOMYCIN
H_2O_2
100μ mole ACETATE
H_2O_2
30μ mole PHOSPHATE
H_2O_2
H_2O_2
ANOXIC

as the sodium salt had no measureable effect on K^+ uptake, while sodium acetate also had little effect. There appeared to be an essential requirement for phosphate to attain sizeable K^+ uptake. Maximum uptake occurred when 5 mM sodium phosphate was present with 10 mM sodium acetate.

Fig. 7 further demonstrates the enhancement of K^+ uptake by addition of acetate and phosphate. In addition it was observed that phosphate and acetate were both required at an extramitochondrial concentration of K^+ of 1.0 mM in order to attain maximum K^+ uptake.

F. Effects of Electron Transport Inhibitors and ATP Addition on Potassium Ion Uptake

To further explore whether respiratory energy was being used to sustain induced K^+ uptake in mitochondria from pea cotyledons, different respiratory inhibitors were used (Table VIII). The concentrations of inhibitors used were those known to be effective with plant mitochondria (Wilson and Hanson, 1969).

The results (Table VIII) were obtained from the same mitochondrial preparation by adding inhibitors and ATP in the second or third cycle of uptake. Succinate or succinate plus rotenone sustained normal K^+ uptake. Rotenone inhibits the first phosphorylation site and hence did not affect succinate utilization. Antimycin A, however, blocks the second site of phosphorylation, hence succinate utilization. As expected, it caused rapid efflux of K^+ into the extramitochondrial solution (Fig. 8) and prevented any further K^+ uptake when H_2O_2 was added (Table VIII).

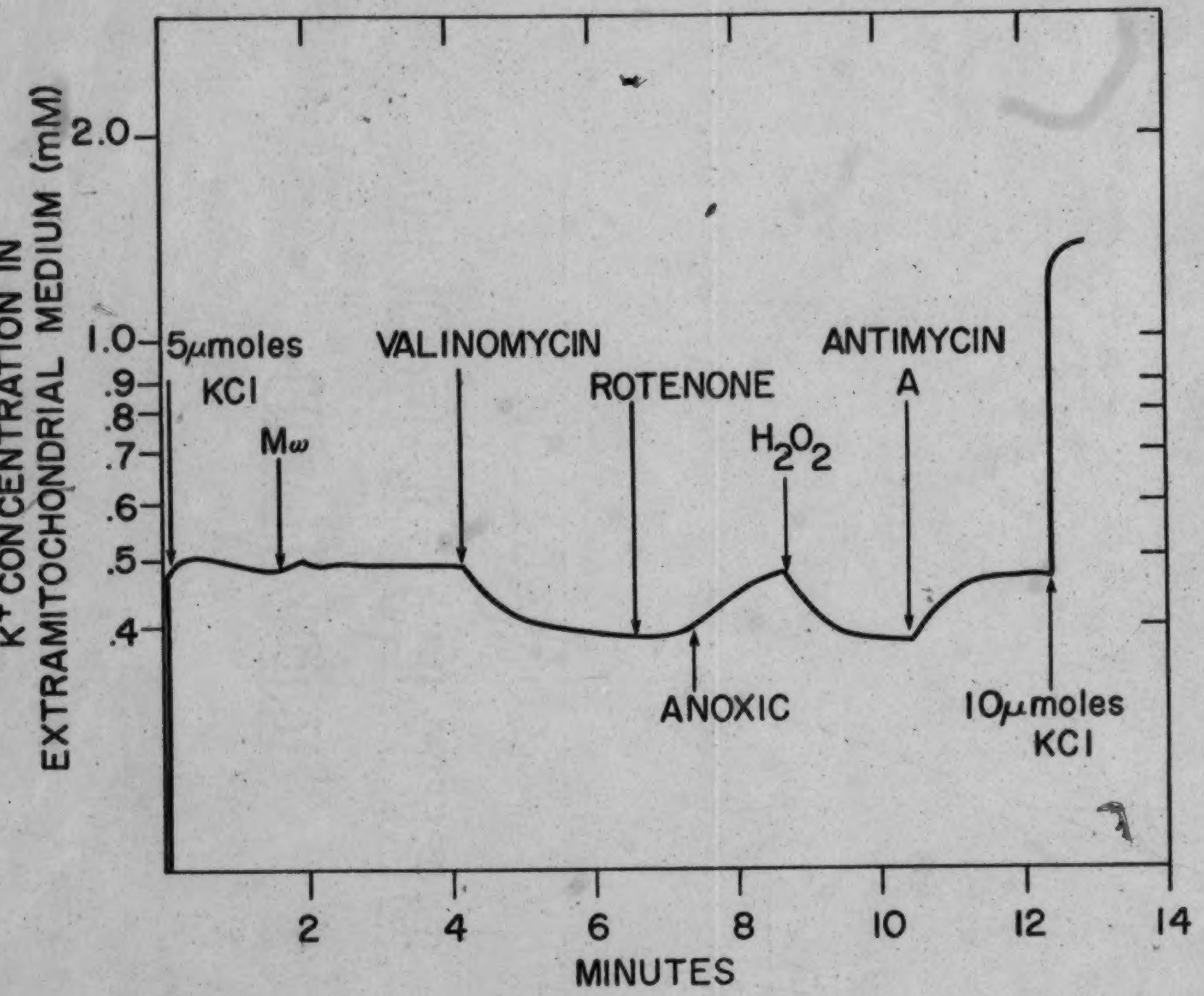
The presence of ATP (1.2 mM) did not sustain any K^+ uptake when

TABLE VIII. Effects of Electron Transport Inhibitors and ATP Addition on Potassium Ion Transport

Reaction Conditions	'Total' K$^+$ Uptake nequiv/mg protein	Rate K$^+$ Uptake nequiv/mg protein/min
1. Reaction buffer without succinate	0	0
2. Reaction buffer with succinate	130	280
3. Treatment 2 (above) + 0.5 µg rotenone per ml mitochondrial suspension	130	290
4. Treatment 2 (above) + 0.5 µg antimycin A per ml mitochondrial suspension	0	0
5. Treatment 2 (above) + 0.5 µg antimycin A per ml + 12 µmoles ATP	0	0
6. Treatment 2 (above) with mitochondria in anoxic conditions + 12 µmoles ATP	0	0

Results are reported for mitochondria isolated from etiolated pea cotyledons six days after imbibition. Assay was done at 25°C with 8.4 mg mitochondrial protein in 10 ml of a reaction buffer consisting of 0.3M mannitol, 10mM sodium acetate, 5mM sodium phosphate, and 20 mM succinate where indicated. The buffer was adjusted to pH 7.2 at 25°C with Tris. Potassium movements were initiated by addition of 0.5 µg valinomycin/ml suspension. Extramitochondrial K$^+$ concentration was 0.5mM.

Fig. 8. Effect of electron transport inhibitors on potassium ion movements. Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. Mitochondria (7.3 mg) were added to 10 ml of a buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate and 5 mM sodium phosphate. The pH was adjusted to 7.2 at 25°C with Tris. Reaction was at 25°C. Respiratory parameters indicated that the mitochondria were intact. Potassium ion movements were initiated with addition of 0.5 μg valinomycin/ml suspension.


K$^+$ CONCENTRATION IN EXTRAMITOCHONDRIAL MEDIUM (mM)
2.0
1.0
.9
.8
.7
.6
.5
.4
5μmoles KCl
Mω
VALINOMYCIN
ROTENONE
ANOXIC
H_2O_2
ANTIMYCIN A
10μmoles KCl
2
4
6
8
10
12
14
MINUTES

ATP was added to mitochondria in the anoxic state or antimycin-treated mitochondria. As will be reported in section III-D, extramitochondrial ATP was reutilized only very slightly by valinomycin-treated mitochondria in anoxic conditions.

G. Effects of Divalent Cations on Potassium Ion Uptake

As Mg^{++} has been shown to be necessary in stimulation of a Mg^{++}-dependent ATPase in mitochondria from pea cotyledons (Phillips, 1971) an investigation of possible effects of Mg^{++} on energy-dependent K^+ uptake was carried out. Calcium ions have been shown to inhibit phosphorylation in animal mitochondria (Lehninger, 1949) and to be accumulated with phosphate in plant mitochondria (Truelove and Hanson, 1966). An H^+ efflux has been associated with the energy-dependent uptake of both cations (see review Pressman, 1970). In the present work, both cations were shown to have an inhibitory effect on the valinomycin-induced K^+ uptake (Table IX). Results were obtained from the same preparation of mitochondria by measuring uptake in consecutive cycles with increasing $MgCl_2$ concentrations.

At a 5 mM concentration, $CaCl_2$ showed greater inhibition than $MgCl_2$ on the 'total' uptake and rate of uptake of K^+. As $MgCl_2$ was of more interest than $CaCl_2$ in the present work, (in view of the effects of Mg^{++} on an ATPase), only very limited study was done on Ca^{++}. With increasing concentration of $MgCl_2$ greater inhibition of the rate and extent of K^+ uptake was noted. A similar inhibition was observed in a different preparation when the extramitochondrial K^+ concentration was 1.0 mM.

TABLE IX. Effects of Divalent Cations on Potassium Ion Uptake

	Total Uptake nequiv/mg protein	Rate of Uptake nequiv/mg protein/min
1. Reaction buffer	160	720
2. Reaction buffer + 2mM $MgCl_2$	130	600
3. Reaction buffer + 5mM $MgCl_2$	100	560
4. Reaction buffer + 10mM $MgCl_2$	74	410
5. Reaction buffer + 5mM $CaCl_2$	80	360

Results are reported for mitochondria samples isolated from etiolated pea cotyledons five days after imbibition. The measurements were made at 25°C in 10 mls of a buffer of the following composition: 0.3 M mannitol, 10 mM sodium acetate, 5 mM sodium phosphate and 20 mM succinate. The pH was adjusted to 7.2 at 25°C with Tris. Extramitochondrial concentration of K^+ was 0.5 mM. Mitochondrial protein was 5.4 mg in 10 ml. Potassium ion uptake was initiated by addition of 0.5 μg valinomycin/ml suspension.

H. Effects of pH on Potassium Ion Uptake

The effects of pH on K^+ uptake were studied on the same preparation by measuring K^+ uptake in consecutive cycles wherein the pH had been adjusted by addition of HCl or unneutralized Tris. Table X indicates that maximum rates of, and 'total', K^+ uptake occur at a pH of 7.2 when extramitochondrial K^+ is 0.5 or 1.0 mM. A pH of 7.2 is the optimum pH for succinate utilization by mitochondria of pea cotyledons (S. S. Malhotra, unpublished results). It was also noted that the K^+ uptake dropped to almost nil by the third cycle of uptake for a mitochondrial preparation in the presence of a buffer of pH 7.8. In addition it was observed that when the buffer pH was adjusted to 7.2 from a pH of 6.2 or 7.8 neither the rate nor the 'total' K^+ uptake increased as expected.

I. Uptake of Potassium Ions by Mitochondria in Differing Extramitochondrial KCl Concentrations

With increases in extramitochondrial KCl concentrations, (up to 2.0 mM) higher 'total' uptake of K^+ occurred, and at a faster rate (Table XI). As the concentration was increased it became increasingly difficult to accurately determine 'total', and rates of uptake of K^+, hence the values reported for a 4.0 mM concentration are less accurate than those reported for lesser KCl concentrations. In each run the KCl concentration was increased in stepwise increments to the KCl concenration indicated and allowed to complete one cycle for each concentration. A duplicate was run for the first preparation and values reported are an average of the duplicates which were in close agreement.

TABLE X. Effects of pH on Potassium Ion Uptake

Extramitochondrial KCl Concentration	pH	K^+ Uptake 'Total' nequiv/mg protein	 Rate nequiv/mg protein/min
1.0 mM	6.2	150	100
	6.6	150	410
	7.2	230	690
	7.8	160	430
0.5 mM	6.2	70	150
	7.2	115	210
	7.8	40	80

Results are reported for mitochondria isolated from etiolated pea cotyledons five days after imbibition. Assay was done at 25°C in a reaction buffer consisting of 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate and 5 mM sodium phosphate. pH was adjusted from 7.2 to the pH indicated with Tris or HCl. Potassium movements were initiated by addition of 0.5 µg valinomycin/ml suspension. Mitochondrial protein was 7.8 mg/10 ml buffer. Respiratory parameters measured at pH 7.2 indicated that the mitochondria were intact.

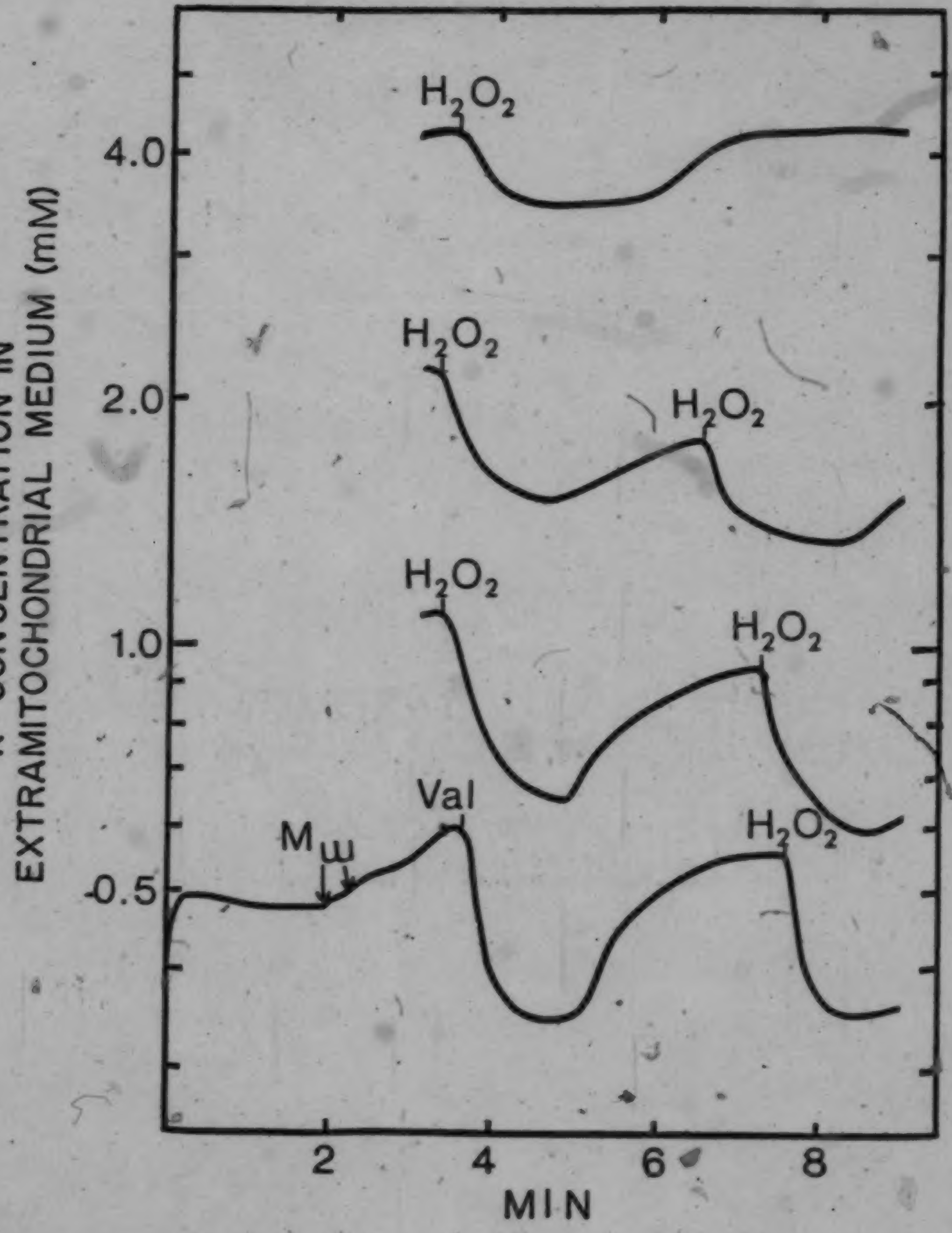
TABLE XI. Potassium Ion Transport in Mitochondria Suspended in Differing KCl Concentrations

	K^+ Movements			
	Mitochondrial Preparation #1		Mitochondrial Preparation #2	
Extramitochondrial K^+ Concentration	'Total' K^+ Uptake nequiv/mg protein	Rate nequiv/mg protein/min	'Total' K^+ Uptake nequiv/mg protein	Rate nequiv/mg protein/min
0.5mM	140	170	110	290
1.0mM	220	480	175	410
2.0mM	270	630	250	670
4.0mM	400	600	300	630

Results are reported for two samples of mitochondria isolated from etiolated pea cotyledons five days after imbibition. Assay was done at 25°C in a reaction buffer consisting of 0.3M mannitol, 20mM succinate, 10mM sodium acetate, and 5mM sodium phosphate. The buffer pH was adjusted to 7.2 at 25°C with Tris. Potassium movements were initiated by addition of 0.5 µg valinomycin/ml mitochondrial suspension. The mitochondrial protein concentration was 6.3 mg/10 ml in the first preparation and 14.3 mg for the second preparation.

Fig. 9. Potassium ion uptake in mitochondria suspended in differing KCl concentrations. Assay conditions are as in Table XI. Results are for the same sample of mitochondria isolated from etiolated pea cotyledons five days after imbition. The traces were obtained by recording cycles of uptake and efflux through periods of aerobic and anoxic conditions as the KCl concentration was increased stepwise. Mitochondrial protein was 14.3 mg/10 ml buffer. Reaction was at 25°C. Potassium movements were initiated by addition of 0.5 µg valinomycin (VAL)/ml suspension for the cycle and H_2O_2 for subsequent cycles.

7


K⁺ CONCENTRATION IN EXTRAMITOCHONDRIAL MEDIUM (mM)
4.0
2.0
1.0
-0.5
H_2O_2
H_2O_2
H_2O_2
H_2O_2
H_2O_2
H_2O_2
Val
M
ω
2
4
6
8
MIN

When mitochondrial protein is increased to 14.3 mg (approximately double that of most runs) very enhanced movements of K^+ is noted (Fig. 9). The advantage of using such a large amount of mitochondrial protein is self-evident, but the capacity of the isolation procedure used ~~did not~~ permit routine use of such a large sample. The figure demonstrates K^+ uptake and subsequent efflux from the same sample at four different concentrations of KCl.

Since the electrode measures change in K^+ activity in a logarithmic mode, the changes in K^+ concentration in the 4.0 mM KCl solution gives much higher rates and 'total' uptake than those for the lowest KCl concentrations, even though the actual potential change is lower at 4.0 mM KCl.

J. Respiration Rates of Mitochondria in the Presence of Valinomycin and Different KCl Concentrations

A direct investigation was carried out on the effect of valinomycin on the respiration rates of mitochondria suspended in buffers with different concentrations of KCl (Table XII). The mitochondria isolated in both preparations showed an RCR of close to 2.8 in the absence of valinomycin. With no added extramitochondrial KCl, inhibition of respiration resulted when valinomycin was added. As the KCl concentration was increased, respiration increased until it approached that of state III respiration when the KCl concentration was 1.0 mM. Respiratory control was evident when ADP was added to mitochondria with respiration rates less than that of the state III rate (extramitochondrial KCl concentration usually 1.0 mM or lower).

TABLE XII. Respiration Rates of Mitochondria in the Presence of Valinomycin and Different KCl Concentrations

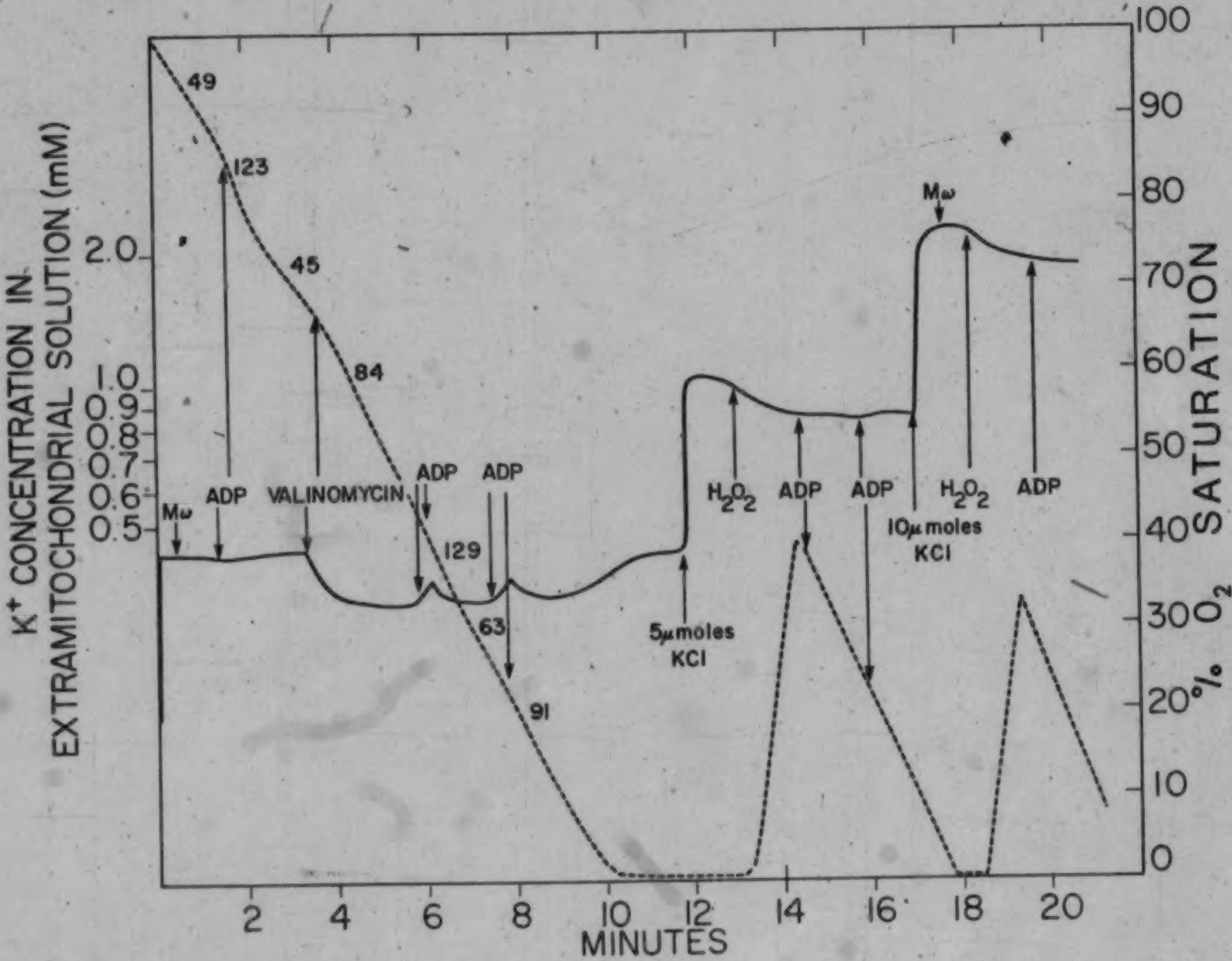
Conditions for measurement	nmol O_2 utilized/mg protein/min	
	Mitochondrial Preparation #1	Mitochondrial Preparation #2
State IV respiration	54	35
State III respiration	150	96
Valinomycin and no added KCl	42	31
Valinomycin + 0.5 mM KCl	101	72
Valinomycin + 1.0 mM KCl	135	86
Valinomycin + 2 mM KCl	147	88
Valinomycin + 5 mM KCl	147	90

Results are reported for mitochondria isolated from etiolated pea cotyledons five days after imbibition. Assay buffer was of the following: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate and 5 mM sodium phosphate. The buffer was adjusted to pH 7.2 at 25°C with Tris. Valinomycin was present at a concentration of 0.5 µg valinomycin per ml buffer. Mitochondrial protein was 0.43 mg/ml buffer for the first preparation and 0.34 mg/ml for the second. Addition of 250 nmol ADP was used to check state III respiration. Reaction was at 25°C.

K. Effects of ADP Addition on Potassium Ion Movements

Fig. 10 shows the apparent competition for respiratory energy between K^+ ion transport and ATP synthesis. In the absence of valinomycin and with the first addition of ADP there was apparently a slight efflux of K^+. The validity of the observation was not investigated further because of the small K^+ change that occurred. After addition of valinomycin a sizeable uptake of K^+ occurred, and the respiration rates approximately doubled. Addition of ADP under these conditions caused a still further increase in respiration rate. Respiratory control was thus observable. At the same time, the K^+ electrode indicated a marked increase in extramitochondrial K^+. Furthermore it was observed in other runs that addition of ADP to mitochondria in the anoxic state did not cause any change in electrode response. Also if ADP was added at the same time as H_2O_2, uptake of K^+ would occur to a certain point, maintain that level and then resume uptake when ADP had been phosphorylated. Also when the extramitochondrial KCl concentration was increased, addition of ADP caused less change in the K^+ tracing (Fig. 10). Later work indicated that ADP was phosphorylated very slowly in the presence of valinomycin and high KCl concentrations (see Section III-B).

Fig. 10. Effects of ADP addition on potassium ion movements at different concentrations of extramitochondrial K^+. Results are for mitochondria isolated from etiolated pea cotyledons. (---) represents % O_2 saturation while (-) is the tracing of K^+ concentration changes. Assay buffer was of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate and 5 mM sodium phosphate. The buffer was adjusted to pH 7.2 at 25°C with Tris. Mitochondrial protein was 9.1 mg/10 ml buffer. Adenosine-5'-diphosphate (250 n mol) was added at each of the times indicated. Reaction was at 25°C.


K⁺ CONCENTRATION IN EXTRAMITOCHONDRIAL SOLUTION (mM)
2.0
1.0
0.9
0.8
0.7
0.6
0.5
100
90
80
70
60
50
40
30
20
10
0
% O₂ SATURATION
2
4
6
8
10
12
14
16
18
20
MINUTES
Mω
ADP
VALINOMYCIN
ADP
ADP
5μmoles KCl
H₂O₂
ADP
ADP
10μ moles KCl
H₂O₂
ADP
Mω
49
123
45
84
129
63
91

III. ATP Synthesis in Isolated Mitochondria from Pea Cotyledons

A. Characteristics of ATP Synthesis

1. Localization of ATP following phosphorylation of ADP

The data (Table XIII) indicate that ADP was phosphorylated completely to ATP under the conditions used. It appeared that as ADP was phosphorylated it was deposited on the outer phase of the mitochondria. Very little ATP could be detected in a pellet of mitochondria that had been removed from the reaction mixture by centrifugation.

2. Reproducibility of ATP measurements

Table XIV demonstrates the reproducibility of ATP measurements determined fluorometrically. It was noted that there was much greater reproducibility in samples when $MgCl_2$ was present as compared to its absence, hence results are reported for reactions done in the presence of $MgCl_2$. When samples from the same mitochondrial preparation were used, analysis gave very similar results. The ADP was phosphorylated very rapidly in the first thirty seconds and then the reaction slowed down. Time course studies of the rate of synthesis are presented in Section III B. In comparisons of samples from different mitochondrial preparations somewhat greater variation is indicated; however, the values reported are still reasonably close. (Slight variations in timing of pipetting could be responsible for some differences.)

B. Time Course of ADP Phosphorylation in Presence and Absence of Valinomycin

It has already been demonstrated that addition of ADP can interfere with the valinomycin-induced K^+ uptake (Fig. 10). In addition,

TABLE XIII. Conversion of ADP to ATP in Mitochondrial Suspensions

mitochondrial preparation	mg mitochondrial protein	n mol ADP added to 1 ml mitochondrial suspension	n mol ATP present in mitochondria following separation from assay buffer	n mol ATP present 1 ml supernatant layer
#1	1.4	125	2	130
#2	2.1	200	4	198
#3	1.1	300	0	305

Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. Mitochondria and ADP added to the mitochondria in a buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate and 5 mM sodium phosphate. Buffer pH was adjusted to 7.2 at 25°C with Tris. Samples were removed from reaction vessel 3 minutes after ADP addition. ATP analysis was as indicated in materials and methods. Reaction was at 25°C.

TABLE XIV. Reproducibility of ATP Synthesis Measurements

Mitochondrial Preparation		ATP as % of added ADP			ADP Phosphorylated/mg protein/min
		30 sec	1½ min	3 min	
Reproducibility in Duplicate Samples					
#1	a	55	92	104	543
	b	55	93	106	543
#2	a	57	106	106	594
	b	58	110	108	610
Reproducibility in Different Mitochondrial Preparations					
#1		55	92	104	543
#2		57	106	106	594
#3		53	102	111	537
#4		51	86	110	535

Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. Mitochondria (each sample was in the range of 1 mg protein) were added to 5 ml of a buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate, 5 mM sodium phosphate, 2.5 mM KCl and 5 mM $MgCl_2$. The pH of the buffer was adjusted to 7.2 at 25°C with Tris. One minute after introduction of the mitochondria to the buffer, 750 n mole ADP was added and one ml samples were removed at the times indicated. Reaction was at 25°C.

valinomycin leads to loss of respiratory control depending on the extramitochondrial concentration of KCl (Table XII). The results of a study of the time course of ATP synthesis in isolated mitochondria in the presence and absence of valinomycin is shown in Fig. 11. The rate of ATP synthesis was much slower in the presence of valinomycin than in the absence. In the absence of valinomycin, the ATP concentration reached 90% of the amount of ADP added after 4 min and was 96% of the ADP added when sampled at 9 min. In the presence of valinomycin the concentration of ATP was only 35% of the amount of ADP nine minutes after the ADP addition. It was calculated that the net rate of ATP synthesis in the absence of valinomycin was 180 n mol/mg protein/min which is much slower than the rates indicated in Table XIV when Mg^{++} was also present. The initial rate of ATP synthesis in the presence of valinomycin and 2.5 mM KCl was only 79 n mol ADP phosphorylated/mg protein/min.

C. Phosphorylation of ADP in the Presence of Valinomycin under Differing KCl Concentrations

The effects of different extramitochondrial KCl concentrations on the level of ATP synthesized in the presence of valinomycin, is shown in Fig. 12. At low concentrations of KCl (1.0 mM and lower) there were only slight effects on the rate and level of ATP synthesized compared to the control without valinomycin. As the external KCl concentration was increased greater effects on the phosphorylation of ADP became apparent. It was noted that some variation occurred in the final levels of ATP at each KCl concentration in different runs. This was likely a result of slight variations in the intactness of the mitochondria.

Fig. 11. Time course of ADP phosphorylation in the presence (--) and absence (-) of valinomycin at an extramitochondrial KCl concentration of 2.5 mM. Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. Mitochondria (2.6 mg) were added to 11 ml of a buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate and 5 mM sodium phosphate. The pH of the buffer was adjusted to 7.2 at 25°C with Tris. Samples of 1 ml were removed at the times indicated. Valinomycin at a concentration of 0.5 μg/ml of assay buffer was added 1 min prior to addition of ADP (2,000 nmol). Reaction was at 25°C.

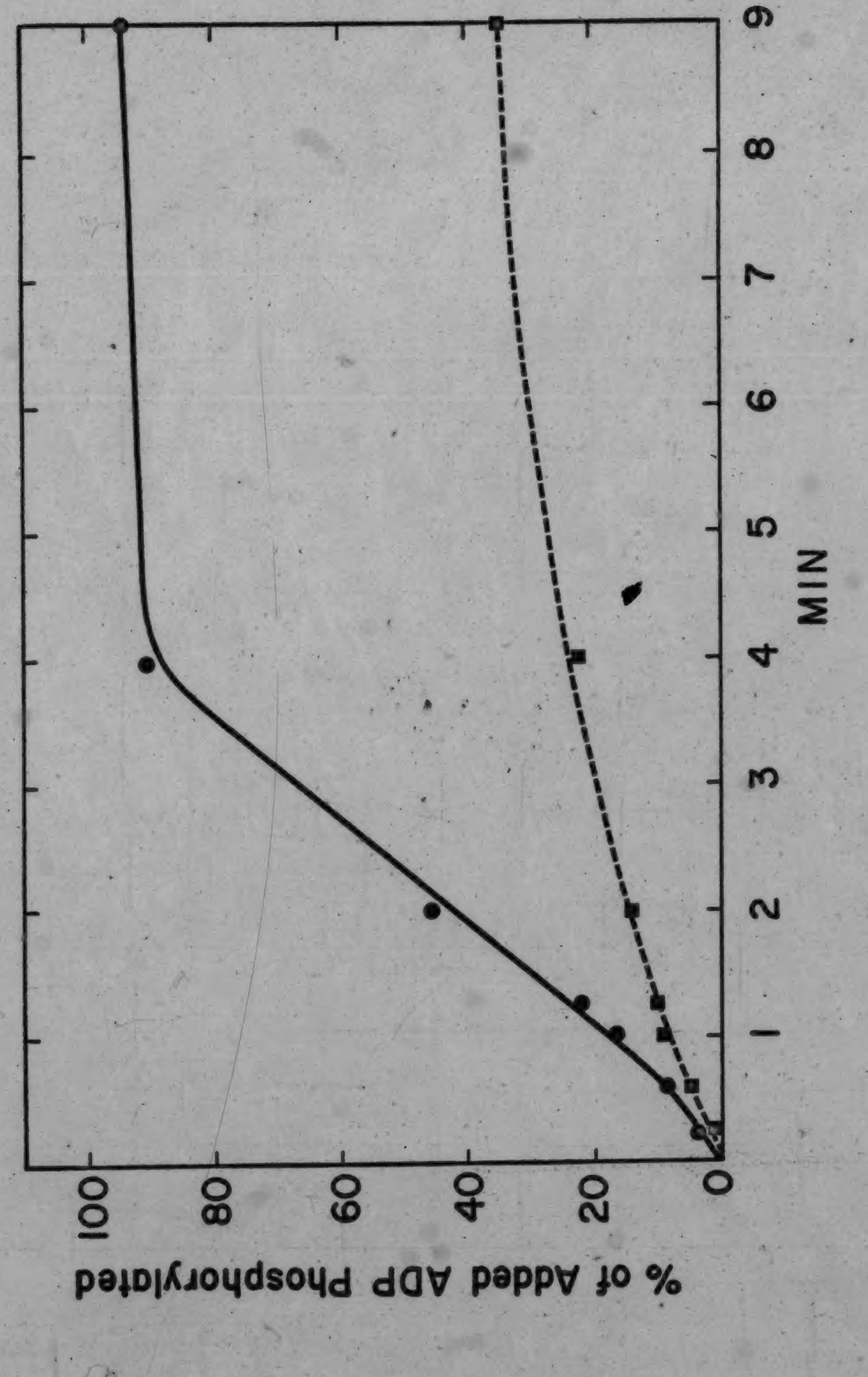
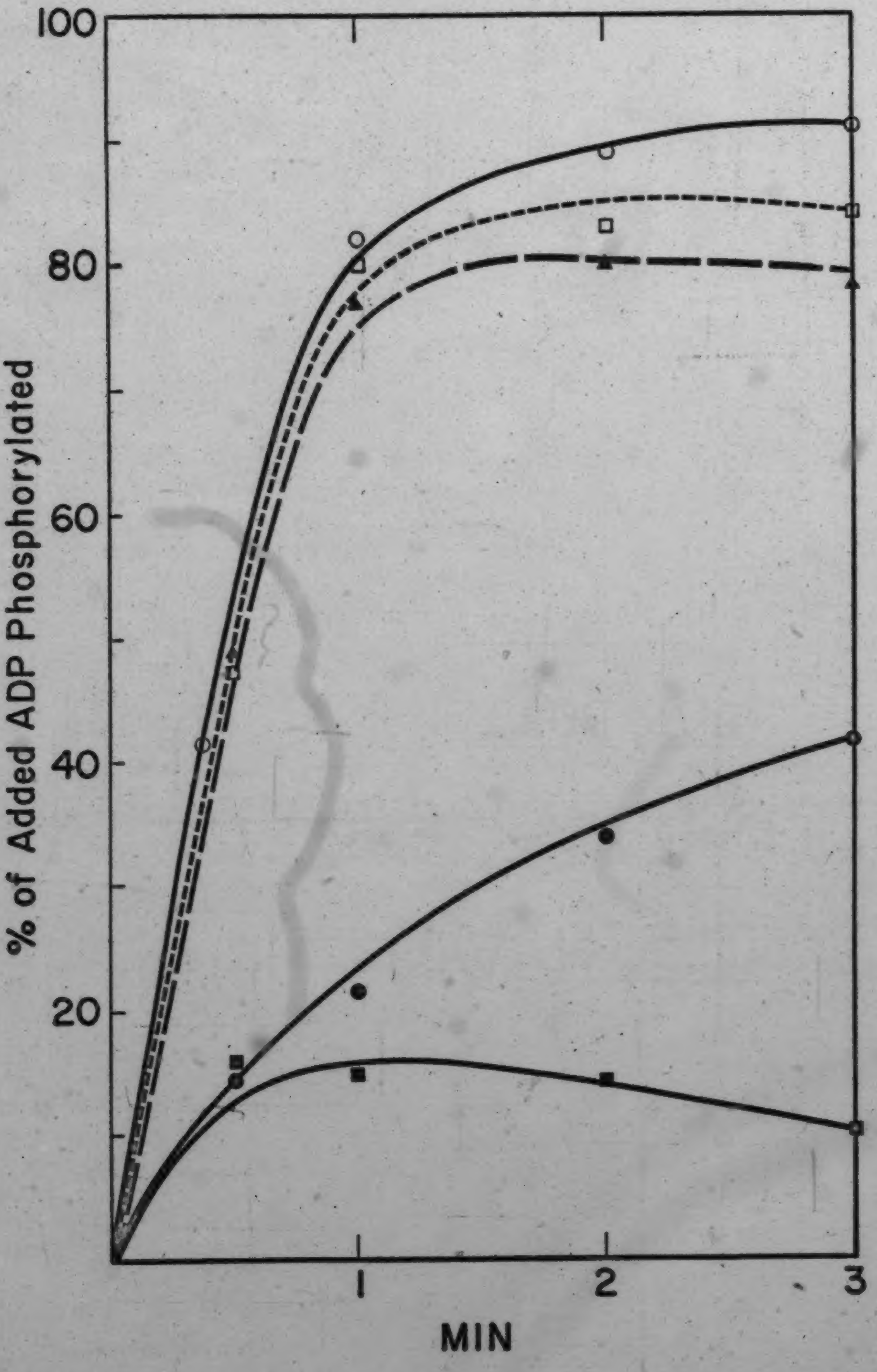

% of Added ADP Phosphorylated
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
MIN

Fig. 12. Phosphorylation of ADP in the presence of valinomycin at different levels of KCl. Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. The mitochondrial suspension (2.2 mg) was added to a 5 ml of reaction buffer of the same composition as in Fig. 11. Potassium chloride concentration was as indicated. Adenosine diphosphate (750 nmol) was added to the assay buffer one minute after addition of valinomycin. Reaction was at 25°C.

Control (○—○) no valinomycin with 2.5 mM KCl; valinomycin with 0.5 mM KCl (□—□); valinomycin + 1.0 mM KCl (▲—▲); valinomycin + 2.5 mM KCl (●—●); valinomycin + 5 mM KCl (■—■).



% of Added ADP Phosphorylated
100
80
60
40
20
1
2
3
MIN

D. Phosphorylation of ADP and Reutilization in Anoxic Conditions

It was observed in previous work (Table VIII) that ATP could not substitute for respiratory energy in sustaining K^+ uptake under conditions where respiratory energy was unavailable. Thus it was desireable to determine what changes in ATP occur under these conditions.

Fig. 13 shows the formation of ATP throughout an aerobic phase when ADP was present and changes that occurred in ATP levels during an anoxic phase. In the absence of valinomycin ATP levels dropped rather quickly initially and then began to level out. When valinomycin was present, however, the reutilization of ATP occurred to only a very moderate extent. Reports in the literature for animal mitochondria indicate that added ATP can substitute for respiratory energy in sustaining K^+ transport when respiratory energy is not available (Harris et al., 1966). The present observations do not agree with this report. The ATP concentration for mitochondria entering the anoxic state in the absence of valinomycin was 184 μM while the concentration of ATP was 74 μM for mitochondria entering the anoxic state in the presence of valinomycin.

E. Effect of Presence of Mg^{++} on ATP Synthesis

The presence of $MgCl_2$ has been shown to have an inhibitory effect on K^+ ion uptake. Investigations of the effect of $MgCl_2$ on the rate of ATP synthesis (Table XV) show a much enhanced rate of synthesis in the presence of $MgCl_2$, particularly in the assay 30 seconds after ADP addition. As the $MgCl_2$ concentration is increased to 25 mM and higher, the rate of synthesis is slowed down. In the presence of valinomycin, $MgCl_2$ has a stimulatory effect on ATP synthesis as well.

Fig. 13. Phosphorylation of ADP in aerobic conditions and reutilization in anoxic conditions in presence (--) and absence (-) of valinomycin. Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. A suspension of mitochondria (2.7 mg protein) was added to 10 ml of a buffer of the same composition as Fig. 11. Extramitochondrial KCl concentration was 2.5 mM. Addition of 2,000 nmol ADP was made two minutes after addition of mitochondria. Valinomycin (0.5 μg/ml suspension) was added, where indicated, one minute after mitochondrial addition. Reaction was at 25°C.

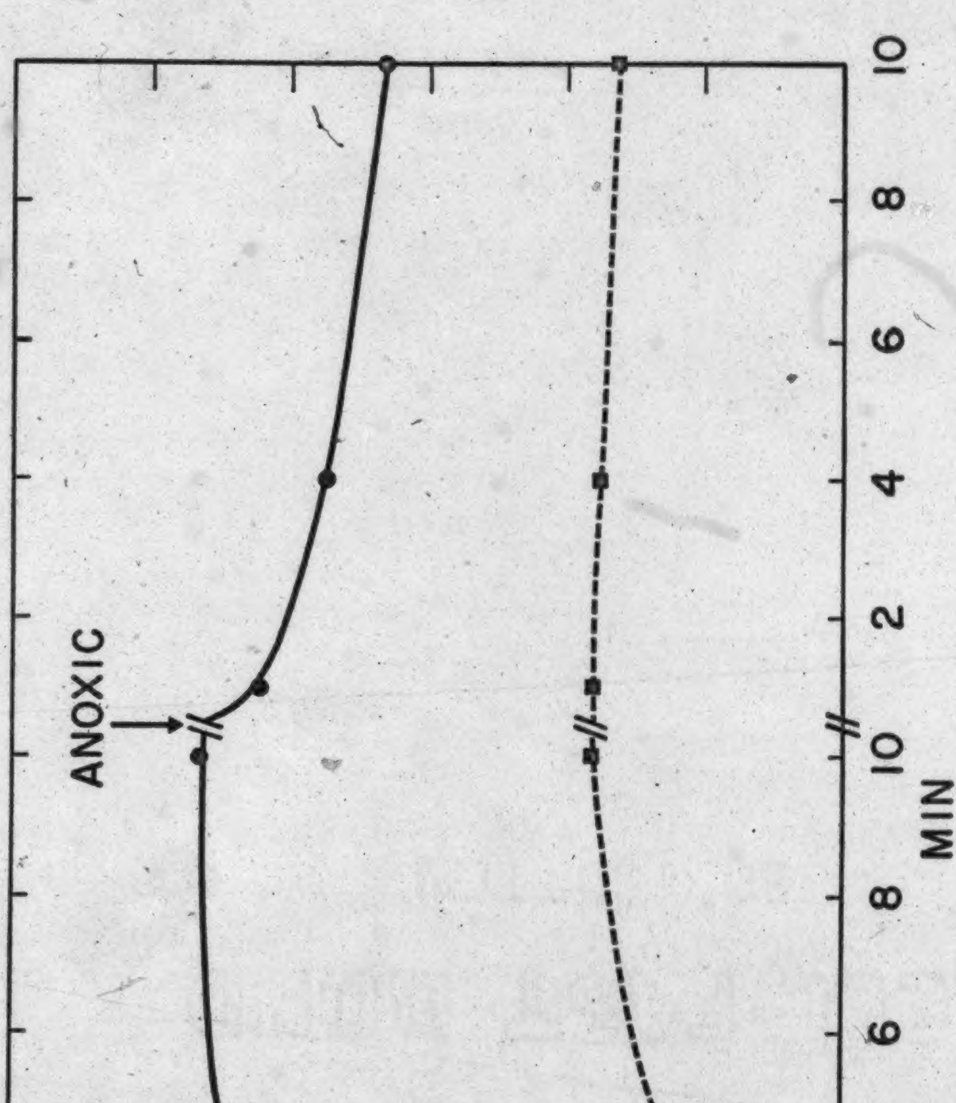

ANOXIC
ATP as % of Added ADP
100
80
60
40
20
MIN
2
4
6
8
10
2
4
6
8
10

TABLE XV. Effect of Presence of $MgCl_2$ on ADP Phosphorylation in Presence and Absence of Valinomycin

Absence of Valinomycin	ATP as % Added ADP			Rate ATP Synthesized/min/mg protein
$MgCl_2$	30 sec	1½ min	3 min	
0 mM	28	73	98	290
10 mM	55	87	103	570
25 mM	51	84	107	530
50 mM	46	69	98	475

Presence of Valinomycin		ATP as % Added ADP		Rate ATP
KCl	$MgCl_2$ (5 mM)	30 sec	3 min	Synthesized/min/mg protein
1.5 mM	+	31	89	332
	-	26	65	278
2.5 mM	+	14	39	150
	-	8	16	86
5.0 mM	+	16	21	171
	-	9	12	96

Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. Mitochondrial protein (1.4 mg protein) was added to 5 ml of a buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate, 5 mM sodium phosphate and $MgCl_2$ and KCl concentrations indicated. The buffer was adjusted to pH 7.2 at 25°C with Tris. Valinomycin was added where indicated in the concentration of 0.5 mg/ml suspension. Adenosine diphosphate was added in the amount of 750 nmol. Reaction was at 25°C.

F. Effect of Timing of Valinomycin Addition on ATP Synthesis

A further investigation into the competitive aspects of ATP synthesis and K^+ ion uptake was done to show that rates of ATP synthesized when the K^+ gradient had already formed (i.e. valinomycin was added one minute prior to ADP addition). This was compared with the rates of ATP synthesis when valinomycin was added at the same time as ADP was added. In the latter case the K^+ gradient would not be preformed and phosphorylation of ADP would compete with the formation of the K^+ gradient for respiratory energy. Table XVI shows that ADP was phosphorylated at the same rate regardless of the presence or absence of valinomycin at an extramitochondrial KCl concentration of 0.5 mM or 1.0 mM. When the extramitochondrial concentration of KCl was increased to 2.5 mM, timing of addition of ADP following valinomycin had a very pronounced effect.

When ADP was added 1 minute after valinomycin addition when the K^+ gradient had already formed, ATP was synthesized much faster than when ADP was added simultaneously with valinomycin. Phosphorylation of ADP was slow at first but the total phosphorylation after three minutes was approaching the level of ATP in the early valinomycin addition.

TABLE XVI. Effect of Timing of Valinomycin Addition on ATP Synthesis

K^+		% ADP Converted to ATP		
		30 sec	1½ min	3 min
0.5 mM	No valinomycin	28%	73%	98%
	Valinomycin added 1 min prior to ADP addition	30%	72%	92%
	Valinomycin added simultaneously with ADP	28%	70%	94%
1.0 mM	No valinomycin	33%	75%	98%
	Valinomycin added 1 min prior to ADP addition	32%	44%	74%
	Valinomycin added simultaneously with ADP	33%	47%	72%
2.5 mM	No valinomycin	35%	76%	100%
	Valinomycin added 1 min prior to ADP addition	16%	28%	32%
	Valinomycin added simultaneously with ADP	7%	12%	27%

Mitochondria were isolated from etiolated pea cotyledons five days after imbibition. Mitochondrial protein (1.5 mg protein) was added to 5 ml of a buffer of the following composition: 0.3 M mannitol, 20 mM succinate, 10 mM sodium acetate, 5 mM sodium phosphate and KCl concentration as indicated. The buffer was adjusted to pH 7.2 at 25°C with Tris. Valinomycin (0.5 μg/ml suspension) was added where indicated one min after mitochondrial addition. Reaction was at 25°C.

DISCUSSION

I. Preparation of Mitochondria by Zonal Techniques

Density gradient techniques are of immense value in obtaining a mitochondrial preparation that is relatively free from contamination by other sub-cellular organelles. In the present work the technique was worked out for preparation of a purified mitochondrial fraction in a Ti-XIV zonal rotor, with a sample volume of a homogenate of pea cotyledons as large as 250 ml. The procedure used was time-saving in a number of ways: no preliminary centrifugation of the sample for separation of a crude mitochondrial fraction was necessary, the sample was edge-loaded into the rotor in a matter of only a few minutes, and the large capacity of the rotor enabled the isolation of a preparation with 20-30 mg mitochondrial protein.

Subsequent examination of this fraction indicated a high degree of purity in the mitochondrial fraction. Electron micrographs (Fig. 5) show a field of particles isolated from the interface of the 37.5% and 41.5% sucrose steps. Examination shows mainly mitochondria present (either in the condensed or orthodox state) plus a few unidentified particles.

In addition it was found necessary to include 0.1% BSA and adjust the pH of each step in the sucrose gradient to 7.2 (at 2°C) to obtain mitochondria displaying good respiratory parameters. For the present work an RCR value of 2.5 and an ADP/O of 1.7 were considered the minimum values that were acceptable for study of mitochondria in the present thesis. Mitochondria were routinely isolated from etiolated pea cotyledons five days after imbibition since this age yielded mitochondria of the highest respiratory parameters and mitochondria with fully-developed membranes (Solomos et al., 1972). However it was observed by this author that mitochondria isolated from 4 or 6 day old tissue displayed respiratory parameters equal to those of mitochondria from five day cotyledons. It was not necessary in the present work to complete a detailed study of the isolated mitochondrial fraction since Solomos et al. had reported extensively on such studies. Simply, by the zonal ultracentrifugation technique developed, mitochondria were obtained in good yield and with a high degree of purity and good respiratory parameters. The observation of Phillips (1971) that extraneous protein in a crude preparation yields a higher state IV respiration, than a purified mitochondrial preparation is reflected in the values of respiratory parameters obtained in the present work.

Table I shows that the RCR value for a zonal mitochondrial preparation is higher than that for the crude preparation, as is the O_2 utilization on a mg protein basis. The catalase activity has been largely reduced in the mitochondrial fraction and can be detected in high activity in a zonal fraction of higher density. The zonal rotor was a useful technique in obtaining mitochondria for the present work and offered the advantages of capacity, speed, ease in preparation and purified mitochondria.

II. Study of Potassium Ion Movements in Mitochondria

A. Development of Techniques

It was decided to study uptake of potassium ions for examination of ion uptake in mitochondria for a number of reasons. First, since K^+ is a univalent cation little complexing of this ion with other ionic species was expected. Second, K^+ movements have been well-documented in animal mitochondria (e.g. Harris _et_ _al_., 1966). Third, the addition of valinomycin was a means of initiating measureable K^+ uptake, and fourth, ion selective electrodes were available for a sensitive, rapid and continuous monitoring of activity of K^+ ions.

The development of a technique for use of the potassium liquid membrane electrode for measurement of K^+ uptake in mitochondria is new to this field of ion uptake. Although the principle of operation of the electrode depends on the K^+ selectivity of the membrane as induced by valinomycin present in the electrode, any leak of valinomycin from the electrode into the solution could complicate results. However the only problem would lie in observations of spontaneous K^+ uptake. It would be difficult to exclude leakage of valinomycin from the electrode as being responsible for spontaneous uptake. The fact that no consistent spontaneous uptake was observed (with mitochondrial addition) supported the assumption that valinomycin leakage from the electrode would not be a problem. Also the valinomycin molecule is highly lipophilic and would not be expected to be extracted into aqueous solutions. The potassium liquid membrane electrode, on the other hand, contains a water immiscible solvent in which the valinomycin is very soluble. (A description of the electrode is given in Section A-1 of the Appendix).

The use of a K^+ glass electrode for detection of K^+ movements is widely reported in the literature (e.g. Harris _et_ _al_., 1966; Kirk and

Hanson, 1973). However, this electrode does not have as high a specificity for K^+ ions as the liquid-membrane electrode. In early stages of the present investigations, highly erratic responses were obtained with a Beckman K^+ glass electrode. It was not ruled out, however, that a faulty reference electrode or faulty pH meter might have been responsible. The K^+ liquid-membrane electrode was observed to provide highly reproducible values, fast response and a stable potential with very little drift. The fast response was characteristic of the electrode for a small change in K^+ concentration of a solution in which the electrode was equilibrated.

Equilibration of the electrode with the assay solution was carried out prior to addition of mitochondria. With the addition of mitochondria, a slow spontaneous efflux was often noted as detected by an increase in extramitochondrial K^+ concentration. This is in contrast to the results reported by Harris et al. (1966) in animal mitochondria and more recently by Kirk and Hanson (1973) in corn mitochondria. These workers were able to measure a substrate supported uptake of K^+ in the absence of valinomycin. Christie et al., (1965) have reported a loss in K^+ from mouse liver mitochondria incubated at 37°C; this led to enhanced uptake of K^+ in presence of respiratory substrate. With other preparations, the present author observed either small decreases in extramitochondrial K^+ concentrations, or no change. It is probable that isolation conditions and internal mitochondrial K^+ concentration are the major factors determining whether K^+ will be taken up or efflux will occur upon addition of mitochondria to an assay buffer containing respiratory substrate. It is probable that swelling was occurring when mitochondria were added to the succinate medium since Wilson et al. (1969) have shown that an active swelling of mitochondria occurs in the presence of acetate salts. This swelling was independent of the cation present. Since Tris was used in adjusting the pH of the assay buffer it was possible that $Tris^+$ was being actively accumulated with acetate to a much greater extent than K^+ at the low extramitochondrial K^+ concentration used. A significant uptake of salt would result in a decreasing ionic strength such that the potassium electrode would appear to record K^+ efflux. Such a possibility does not seem realistic because of the relatively high ionic strength of the buffer. Addition of valinomycin caused a large and rapid uptake of K^+ such that if $Tris^+$ or other cations had been accumulated to any extent prior to valinomycin addition a rapid exchange of $Tris^+$ for K^+ would have had to occur.

B. Measurement of K^+ Uptake

As demonstrated in Fig. 5, addition of valinomycin to a mitochondrial suspension induced the mitochondria to rapidly accumulate K^+, and caused a concomitant increase in oxygen utilization. These observations are consistent with those reported in the literature for animal mitochondria (e.g. Harris et al., 1966; Rossi and Azzone, 1970). Recent work on mitochondria from bean and cauliflower (Wilson et al., 1972) and mitochondria from corn (Kirk and Hanson, 1973) indicate that swelling occurs with the valinomycin induced K^+ uptake. The uptake of K^+ that was observed reflect rather large concentration gradients formed since the total mitochondrial volume is only a fraction of the 10 ml assay volume. From a hypothetical standpoint it is conceivable that the mitochondria could have a K^+ gradient of the order of 20 mM inside/1 mM outside the mitochondria. This is assuming a rough estimate of the mitochondrial volume is 1/100 of the assay volume (10 ml) and a concentration change of 0.2 mM occurred in a 1.0 mM K^+ solution as a result of uptake. It is only the intention of the author to point out that a 0.1 or 0.2 mM change in concentration of K^+ in the extramitochondrial medium reflects a sizeable uptake by the mitochondria. Rossi and Azzone, (1969) estimate that the ratio of K^+ concentration inside to the K^+ concentration outside is as high as 1600, corresponding to a potential difference of 192 mv. On the other hand, the H^+ gradient is probably only a fraction of a pH unit. Cockrell et al. (1966) estimate that the K^+ gradient is only 16:1, which is still rather sizeable.

As valinomycin causes an increase in membrane permeability to K^+ as well as facilitating an energy dependent uptake of K^+ (Pressman, 1970), it is reasonable to think that an efflux of K^+ had occurred during the uptake process as well. This would be predicted from the observations that the rate of oxygen utilization remains elevated even after the gradient has formed. Furthermore, the gradient is dissipated very readily once respiratory energy is blocked. Fig. 6 shows that the uptake and efflux process is entirely reversible, and dependent on oxygen concentration in the assay solution. Also after addition of KCl to the extramitochondrial solution there was a very rapid change in potential, so that the traces of K^+ uptake and efflux that extend over 1-2 minutes may be assumed to reflect very closely the actual uptake and efflux of K^+ that took place.

The cycles of uptake and efflux were highly reproducible in the first three cycles and this provided a tool for study of K^+ uptake. Even with the use of the zonal rotor, the amount of mitochondrial protein isolated for a study of K^+ transport was not adequate. By use of the cycles of uptake and efflux, effects of different variables or compounds on the subsequent rates of uptake were examined. The tool is, of course, limited in that only cumulative additions can be studied with this method.

The effects of concentration of valinomycin (Table IV) on induced K^+ uptake was studied at an extramitochondrial KCl concentration of 0.5 mM. The highest 'total' uptake and fastest rate were obtained when the valinomycin concentration was 0.5 µg/ml suspension. This concentration was much higher than the levels reported in the literature. Harris _et al_., (1966) used a concentration of 0.05 µg/ml mitochondria and Kirk and Hanson, (1973) report using a level of 0.1 µg/ml. It was observed in later work in this dissertation that a much lower level of valinomycin (0.1 µg/ml) could fully release respiratory control when the extramitochondrial K^+ concentration was 5 mM. (It did not have this effect at K^+ concentrations of 0.5 mM and lower, which were often used for studies in this dissertation.) In addition to the K^+ concentration it is very likely that the quantity of mitochondrial protein used would also be a factor in the level of valinomycin that can sustain maximum transport values. So that the valinomycin concentration would not be a limiting factor, a level of 0.5 µg valinomycin/ml assay buffer was routinely used.

The 'total' uptake of K^+ and rates observed throughout this work compare favorably with those reported for animal mitochondria (e.g. Harris, et al., 1966) and most recently for corn mitochondria (Kirk and Hanson, 1973).

C. Characteristics of, and Conditions for, K^+ Uptake

In early investigations, before good mitochondrial preparations were routinely obtained, it was noted that fairly large variations in K^+ uptake occurred among different preparations. An investigation of K^+ uptake in mitochondria of different degrees of intactness indicated that no uptake could occur in loosely coupled mitochondria. This observation eliminated any possibility of protein absorption of K^+ being responsible for the K^+ uptake observed. Thus in all subsequent experiments respiratory parameters were routinely monitored prior to use of mitochondria in K^+ uptake experiments. An RCR level of 2.5 was selected as the minimum RCR that would be acceptable for use of the mitochondria in K^+ measurements. Nearly all preparations had an RCR of 2.5 or better in the second cycle of ADP addition and as demonstrated in Table 5 differed little in K^+ uptake values from preparations showing an RCR of 3.0 or better.

Since it is not yet possible to prepare uniform mitochondria in different runs, comparisons of effects of different treatments were made only among mitochondria from one preparation. A minimum of three runs was made for each variable tested to verify trends.

The "uncoupling" ability of valinomycin was first reported by McMurray and Begg, (1959), but later Pressman (1963) showed that in the presence of valinomycin an energy-dependent ejection of protons occurs and that K^+ moves counter to the protons. This demonstrated that valinomycin was not acting as a normal uncoupler (Pressman, 1970). In the present experimental work no pH changes were detected. However, the high buffering capacity of the solution (which was 20 mM in succinate, and approximately 20 mM in Tris) might be expected to mask any pH changes. As it is of value to maintain a stable pH when measuring K^+ uptake, the lack of detectable H^+ efflux proved a bonus in this respect. Sodium ion concentration was not routinely monitored since the concentration of Na^+ was of the order of 20 mM and any change of 0.1 or 0.2 mM in this concentration would be barely detectable.

Succinate was employed as respiratory substrate throughout the present work on measurement of K^+ uptake. Harris et al., 1966, reported slightly higher uptake with a malate-glutamate substrate compared to succinate. Kirk and Hanson (1973) used NADH as substrate since it is not a permeant anion as is succinate. In the present work succinate produced the largest K^+ uptake and fastest movement (Table 6), as well as allowing faster movement through the cycles of uptake and efflux because of the higher O_2 utilization.

Although no intensive studies have been done on exchange-diffusion of anions in plant mitochondria the possibility exists that succinate can move more readily in a K^+/succinate symport mechanism than the other anions tested. Harris (1968) showed that intramitochondrial succinate increases greatly during valinomycin-induced K^+ uptake in rat-liver mitochondria.

The need for the presence of anions other than succinate for sizeable uptake of K^+ is demonstrated in Table VII and Fig. 8. Although other workers (Harris et al., 1966; and Kirk and Hanson, 1973) did not include phosphate salts in the assay buffer (using acetate instead), phosphate was essential to establishment of a high total uptake and a fast rate of uptake in the present work. Wilson et al., (1973) reported that the fastest and greatest swelling of plant mitochondria, as induced by valinomycin, occurred in the presence of phosphate and acetate salts. In the present work phosphate (added as the sodium salt) was absolutely required for a fast rate and a sizeable uptake of K^+ to occur. Sodium acetate (10 mM) enhanced this rate and 'total' uptake so that both salts were included throughout the experimental work. Wilson et al. (1969) studied active swelling of corn mitochondria in the presence of acetate and showed this to be independent of the cations added. It would thus appear that a great proportion of the K^+ uptake observed is in a K^+/anion symport mechanism and in view of the reports of Harris et al., (1966) and Kirk and Hanson (1973) a very small proportion of the K^+ moves in a K^+/H^+ antiport mechanism.

An investigation into the effects of respiratory inhibitors on K^+ uptake (Table VIII) showed that antimycin A caused a very rapid efflux of K^+ and hence abolished the K^+ gradient. Subsequently, ATP was added but it did not sustain any K^+ uptake. This is in contrast to results reported by Harris et al. (1966) with rat liver mitochondria, who showed an ATP-sustained K^+ uptake in the absence of respiratory energy. Christie et al. (1965) showed that Mg^{++} is a necessary cofactor for the ATP-energized uptake of K^+ by K^+-depleted mitochondria (from rat liver). The latter work was done in the absence of valinomycin, however. In the present work, added ATP (1.2 mM) could not substitute for respiratory energy in driving K^+ uptake.

The effects of Mg^{++} and Ca^{++} on K^+ uptake were investigated. Both cations substantially reduced the 'total' uptake as well as the rate of K^+ uptake (Table IX). Harris et al. (1966) have also reported a decrease in the valinomycin-induced rate of K^+ uptake and its 'total' uptake in the presence of those ions. Also, it was found that Ca^{++} had a relatively greater inhibitory effect than Mg^{++} on the K^+ uptake, which is in agreement with the results of Harris and co-workers (1966). Since both cations are known to be actively accumulated and are involved in energy-dependent reactions (see review Pressman, 1970), the present results were not unexpected.

The optimum pH for K^+ uptake was found to be 7.2, which corresponds to the pH optimum for succinate oxidation in mitochondria from pea cotyledons (S. S. Malhotra, unpublished results). Harris et al. (1966) report an optimum pH value of 7.2 for 'total' K^+ uptake in rat liver mitochondria, but a pH of 6.36 sustained the fastest rate. In the present work the fastest rate of K^+ uptake was at pH 7.2. In addition to lower uptake values, the K^+ uptake ability of the mitochondria was lost very quickly at pH 7.8. A very rapid deterioration in K^+ uptake ability occurred at this pH such that by the third cycle of K^+ uptake, almost negligible uptake was observed. Since mitochondria that have lost their intactness display a very poor ability for K^+ uptake, it is possible that a pH of 7.8 rapidly caused a loss of mitochondrial intactness.

The concentration of KCl in the extramitochondrial solution is an important factor in determining the rate and extent of K^+ uptake. At successively higher concentrations of KCl, a greater uptake of K^+ occurred (Fig. 9). Also there was good repeatability of the different values of uptake for differing KCl concentrations.

Since 'total' K^+ uptake and rate of uptake were smaller at a low extramitochondrial K^+ concentration it was assumed that formation of a potassium gradient at a low KCl concentration would be less of a drain on respiratory energy than when it was formed at a higher KCl concentration. If a low KCl concentration required a lesser amount of energy for maintenance of the K^+ gradient, it was thought that examination of other energy-dependent reactions at different KCl concentrations might reveal relationships of K^+ uptake to other energy-dependent reactions.

The effects of valinomycin, and different KCl concentrations, on respiration rates of mitochondria were investigated next. Recently, Kirk and Hanson (1973) have reported an RCR value of 2.8 for NADH oxidation in corn mitochondria in the absence of valinomycin and no added KCl, compared to 2.0 in the presence of valinomycin. Furthermore the RCR dropped to 1.25 when 1 mM KCl was added. Wilson _et al_. (1972) showed a much increased rate of respiration of bean and cauliflower mitochondria with addition of valinomycin to a solution that was 10 mM in KCl. Also no respiratory control was detected when ADP was added.

In the present work, the extramitochondrial concentration of KCl was critical to the respiratory activity in the presence of valinomycin (Table XII). In the absence of added KCl respiration was inhibited somewhat (compared to the normal state IV res[illegible]on). It is possible that with the high levels of valinomycin used, a direct effect of the presence of valinomycin in the membrane on the respiratory chain components may have occurred and this may have accounted for a decrease in respiration. When the KCl concentration was increased to 0.5 mM, respiration approximately doubled over that of the normal state IV respiration. (Additional observations indicated that if ADP was added at this time, respiratory control was still present). When the KCl concentration was increased to 1.0 mM, respiration rate increased to approximately equal that of the state III rate. Addition of ADP at this time did not show a further increase in respiration so that no respiratory control was evident. The results are consistent with less energy being required for the energy-dependent uptake of K^+ at low extramitochondrial KCl concentrations. (There were significantly lower respiratory rates for mitochondria in low KCl concentrations than in high KCl concentrations.)

D. Effects of ADP Addition on Potassium Ion Movements

Other laboratories have reported that valinomycin addition to mitochondria in an anoxic state can lead to a downhill loss of a K^+ gradient. This efflux of K^+ can be used for driving ATP synthesis (Cockrell *et al.*, 1967; Rossi and Azzone, 1970). The amount of ATP synthesized, however, is only about 1/10 of the K^+ efflux on a n mol basis. Later evidence (Cockrell, 1972) indicates that the efflux of K^+ gives rise to a membrane potential (or possibly a H^+ gradient) and this drives ATP synthesis.

In the present investigation, addition of ADP to valinomycin-treated mitochondria caused an efflux of K^+ (Fig. 10). It is known that ADP causes a partial reversal in the energized swollen state of mitochondria

(Packer, 1960, 1961) and that following ADP conversion to ATP, reswelling occurs. It is possible that a reversal of the swollen state is taking place in valinomycin-treated mitochondria during ADP phosphorylation and that this causes K^+ efflux. Alternatively energy that was being used for K^+ uptake is now being preferentially used for ATP synthesis, and with less energy available for uptake, a net K^+ efflux occurs. Still another possibility is that enhanced K^+ efflux (in the presence of ADP) is being used for driving ATP synthesis, possibly through reversal of a K^+-ATPase.

As the external KCl concentration was raised (also Fig. 10) less efflux of K^+ was detected at higher concentrations. This could indicate that either no efflux occurred at the higher KCl concentration or less efflux occurred, but lack of sufficient sensitivity at the higher KCl concentration failed to detect this. When ADP and H_2O_2 were added simultaneously, uptake of K^+ occurred to a certain point, (levelled off) and then resumed when ADP had been phosphorylated. This is further indication that at low extramitochondrial K^+ (0.5 mM) ADP phosphorylation was preferential to K^+ ion uptake to a certain extent, at least. Table XVI indicated that the rate of net ATP synthesis was not affected at low extramitochondrial K^+ concentrations when the ADP was added prior to build-up of a K^+ gradient or after the gradient had formed. At a higher KCl concentration (2.5 mM), however, ADP was more rapidly phosphorylated if the K^+ gradient was already formed. There was competition for energy between the potassium uptake process and ADP phosphorylation. At low extramitochondrial KCl concentrations, however, ADP phosphorylation was preferential to K^+ uptake. The evidence leads to the suggestion that K^+ transport and ATP synthesis are alternative processes for utilization of respiratory energy in pea cotyledons. At low KCl concentrations there was preferential phosphorylation of ADP compared to K^+ uptake but at higher extramitochondrial KCl concentrations, this preference was lost. This may have been caused by a direct effect of valinomycin and K^+ on the reverse ATPase. An increased ATPase activity has been detected by Rossi and Azzone (1970).

III. ATP Synthesis in Isolated Mitochondria from Pea Cotyledons

A. Characteristics

Since ATP synthesis is thought to occur through reversal of an ATPase (Mitchell, 1970) and increased ATPase had been detected in the presence of valinomycin, an investigation of ATP synthesis in pea cotyledon mitochondria was done. The data in Table XIII show that ADP was completely phosphorylated and was largely detectable in the extramitochondrial solution in the absence of valinomycin. This was to be expected in view of the exchange-diffusion theory postulated for transport nucleotides, anion substrates, and other anions in mitochondria (see Chappell, 1968).

The carrier for an ADP-ATP exchange-diffusion reaction ~~has been~~ deduced by the action of atractyloside. Atractyloside blocks the phosphorylation of exogenous ADP but not the phosphorylation of intramitochondrial ADP. This and other observations led to the conclusion that a molecule of ADP enters the mitochondrion only in an exchange for a molecule of ATP (Klingenberg & Pfaff, 1966). Although this work has been done only on animal mitochondria, it is reasonable to expect that such mechanisms are likely present in plant mitochondria.

The fluorometric method employed was very sensitive and able to detect levels of ATP lower than 1 n mol in 4 ml. In addition, duplicate samples demonstrated highly reproducible values for rates of ATP synthesis (Table XIV).

The variation between preparations was much more pronounced as is variations in nearly all parameters in different mitochondrial preparations. The variation in ATP synthesis was reduced in the presence of $MgCl_2$. Hence the results in Table XIV were obtained in the presence of $MgCl_2$.

B. Valinomycin Effects on ATP Synthesis

The increased rate of respiration in the presence of valinomycin masked the phosphorylation of ADP as deduced by changes in oxygraph tracings. Therefore, a study was made of the effects of valinomycin on net ATP synthesis. At an external KCl concentration of 2.5 mM the presence of valinomycin greatly slowed the rate of ATP synthesis compared to ATP synthesis in the absence of valinomycin (Fig. 11). The level of ADP phosphorylated apparently reached a plateau in the presence of valinomycin. An explanation could be that valinomycin was directly affecting the ATPase for ADP phosphorylation, or perhaps valinomycin had an enhanced uncoupling effect with time. However, Phillips (1971) detected only a small increase in a K^+-ATPase in pea cotyledon mitochondria, even at high KCl concentrations. The slow rate of synthesis of ATP in the early stages of the time course when valinomycin was present could be accounted for by an unavailability of energy as a result of energy being used primarily for maintaining the K^+ gradient.

Fig. 12 demonstrates the effect of different levels of KCl and the ability of mitochondria to phosphorylate ADP in the presence of valinomycin. As the extramitochondrial KCl concentration was increased, there was a decrease in the rate of phosphorylation of ADP. Relatively little effect on ATP synthesis was noted at 0.5 or 1.0 mM KCl (when respiratory control could still be detected) in the presence of valinomycin. Also as was established in the K^+ uptake experiments, much more K^+ is picked up and at a faster rate at higher KCl concentrations. This was interpreted to mean a greater drain on respiratory energy occurred with K^+ transport at higher external KCl concentrations. The slow rate of ATP synthesis at higher KCl concentrations substantiates this.

It is of interest that Rossi and Azzone (1970) have shown a loss of mitochondrial ability for ATP synthesis from the valinomycin-induced dissipation of a K^+ gradient at external KCl concentrations above 2-3 mM. As discussed earlier, an increased ATPase activity was also detected. Fig. 12 further shows there is a decrease in ATP level after an initial maximum at 30 sec. for ADP phosphorylation in the presence of valinomycin and 5 mM KCl. It is possible that ATP synthesis had completely stopped and an ATPase activity was accounting for the decrease.

C. Relationship of ATP Synthesis and K^+ Transport

As ATP has been shown to facilitate K^+ ion uptake in rat-liver mitochondria (Harris et al., 1966) but could not sustain K^+ uptake in the present research, an examination of changes in the levels of ATP for mitochondria that are in the anoxic state was carried out. Fig. 13 demonstrates the formation of ATP during the aerobic phase and its subsequent reutilization in the anaerobic state. A rapid initial decrease followed by a slower linear decrease in ATP level occurred when mitochondria entered an anaerobic state in the absence of valinomycin. Very little reutilization of ATP occurred in the presence of valinomycin. It appeared that in the presence of valinomycin, the energy of ATP hydrolysis was not available for sustaining K^+ uptake, an observation which correlated with that of Table VIII.

As $MgCl_2$ was shown to inhibit the K^+ ion uptake and Mg^{++} has been associated with ATPase activity (Discussion II-C), an investigation of effects of Mg^{++} on ATP synthesis both in the presence and absence of valinomycin was investigated. The presence of Mg^{++} is seen to greatly increase the rate of ATP synthesis (Table XV) at a 10 mM concentration but was inhibitory at higher concentrations. A concentration of 5 mM $MgCl_2$ was just as effective as 10 mM in increasing the rate of synthesis.

In the presence of valinomycin Mg^{++} was also shown to increase the rate of ADP phosphorylated. The effect was particularly enhanced at an external KCl concentration of 2.5 or 5.0 mM. It appeared that Mg^{++} was making energy more available for ATP synthesis or more likely was enhancing the activity of the enzyme, so that it could more readily phosphorylate ADP. In addition, as Mg^{++} had an inhibitory effect on K^+ transport, the use of energy for synthesis of ATP thus occurred.

A final investigation as to alternative use of energy for ATP synthesis or K^+ transport (Table XVI) showed that when valinomycin was added prior to ADP addition (a K^+ gradient was given time to form) there was a greater initial rate of ATP synthesized than when ADP and valinomycin were added simultaneously. This occurred if the concentration was at least 2.5 mM but was not observed when the KCl concentration was 1.0 mM or lower.

Addition of ADP simultaneously with valinomycin (at a KCl concentration of 2.5 mM) resulted in a competition for energy by the two processes such that a slower rate of ADP phosphorylation occurred. At low K^+ concentrations (0.5 or 1.0 mM), essentially no difference was apparent whether the valinomycin was added prior to ADP or simultaneously with ADP. This observation is consistent with the idea that ADP is preferentially phosphorylated at low KCl concentrations but at higher KCl concentrations, the demand for energy cannot sustain both processes, and K^+ transport is preferred.

CONCLUSION

Zonal ultracentrifugation techniques can be used to isolate mitochondria of relatively high purity from pea cotyledons displaying good respiratory parameters. They can be obtained quickly and in good yield.

Use of a liquid membrane potassium electrode was of value in a study of potassium ion transport. This method enabled a fast and continuous monitoring of potassium ion uptake in isolated mitochondria.

The valinomycin-induced K^+ uptake observed in the present work was energy-dependent and influenced by such variables as intactness of mitochondria, substrate, anions present, cations present, and level of extramitochondrial KCl concentration. As the KCl concentration in the external solution was increased (from 0.5 mM to 4.0 mM) an increase in rate of, and 'total', K^+ uptake was observed. In addition, a concomitant increase in rate of respiration occurred. Respiratory control was evident if the extramitochondrial KCl concentration was 1.0 mM or lower. At these concentrations ADP could be phosphorylated to ATP in the presence of valinomycin at a rate equivalent to phosphorylation in the absence of valinomycin. The availability of respiratory energy was not limiting and phosphorylation of ADP appeared preferential over K^+ uptake. At higher concentrations much slower rates of ADP phosphorylation occurred. The demand for energy (as indicated by maximum respiration rates) to maintain the K^+ gradient was so demanding that little energy was available for ATP synthesis. Also it was possible that the high concentration of K^+ in the mitochondria (at high external KCl concentrations) interferes with the reverse ATPase process for ATP synthesis. An influence of valinomycin on ATPase activity has been noted by others. ATP could not substitute for respiratory energy in sustaining K^+ uptake. When ADP was added after the K^+ gradient had already formed (at high external KCl concentrations) more energy was available (as indicated by faster rates) for ATP synthesis.

Mg^{++} was required for a fast rate of synthesis of ATP. It also enhanced this synthesis in the presence of valinomycin and was inhibitory to K^+ uptake.

ATP synthesis and potassium ion transport appear to be alternative processes for use of respiratory energy. Although other explanations can be given for individual observations, none account for all the observations made.

APPENDIX

A-1 Electrodes

a. Reference Electrodes

When several electrodes are used to monitor the same test solution, it becomes necessary to use a common reference electrode to minimize cross interactions between electrodes. The function of a reference electrode is to provide a constant potential against which the potential of the measurement electrode is compared. Usually a reference electrode employs a salt-bridge solution. At the liquid junction between the salt-bridge solution and the test solution; the salt solution slowly flows into the test solution. At the interface of the two solutions a potential difference, the liquid junction potential, is established. However, many factors, such as temperature, stirring, and nature of the salt-bridge solution detract from the stability of the electrode potential. There are various types of reference electrodes with liquid-junctions. The reference element is generally Ag/AgCl or calomel with variations in composition of salt-bridge solution and type of liquid-junction. The latter two factors play a major role in stability of liquid junctions.

The most commonly used salt-bridge solution is saturated KCl. Its greatest advantages as a salt-bridge solution are the following.

1. It is of a well defined composition
2. It minimizes and stabilizes the liquid junction potential
3. It has a low differential temperature coefficient

The major disadvantage of saturated KCl is that the rate of contamination by Cl^- and K^+ is much higher than for solutions of low concentration.

In the present work an investigation of the alternative salt-bridge solutions eliminated the possibilities of:

1. Dilute KCl - There was the problem of maintaining composition

2. Saturated LiCl - The salt might have deleterious effects on mitochondria

3. Double junction-electrode - (where a second salt-bridge solution can be inserted between saturated KCl and the test solution).

The third was deemed unmanageable for the measurements required because of its size. Also the salt-bridge solution does not have the advantages of a saturated KCl salt-bridge.

Alternatively one can control rate of contamination by selection of an electrode with a very slow flow rate. The problem that arises however is that the stability of the liquid junction potential is lost as the flow of salt-bridge decreases. For example a sleeve-junction electrode and an open-liquid-junction electrode exhibit very high stabilities of liquid junction potentials. However, they have very high rates of contamination. A fibre junction electrode was found to exhibit erratic liquid junction potentials and it tended to clog in the presence of proteins. In a porous pin electrode, the contact with test solution is made either by a porous pin, sintered glass disc or a ceramic rod. With it, there was found to be a very slow outflow of KCl and resistance to the "Tris buffer" effect*. An A. H. Thomas combination electrode with a ceramic junction Ag/AgCl reference was employed in the research reported here. It exhibited a stable liquid-junction potential and the outflow of KCl was found less than

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*Tris buffer effect is caused by a reaction that takes place between Tris and certain components of various reference electrodes resulting in an erroneous pH reading.

1 μl saturated KCl per hour.

b. K^+ electrode

There are two basic types of K^+ selective electrodes. The first type developed was the glass membrane type, which is identical to the glass pH electrode except selectivity has been conferred upon it by changing the relative composition of different silicates present in the glass bulb. This type however is not entirely selective to K^+ as other ions such as Na^+, H^+, Ag^+ cause erroneous readings.

The liquid membrane K^+ electrode developed by the Orion corporation exhibits an extremely high selectivity for potassium over other cations. The selectivity constants for the Model 92-19 potassium electrode as reported by Orion are: Cs^+, 1.0; NH_4^+, 0.03; H^+, 0.01; Ag^+, 0.001; Na^+, 0.002; and Li^+, 0.001.

A water immiscible solvent (which is 5-10% in valinomycin) is placed in the electrode body such that a lipophilic membrane is saturated with the solution. The K^+ in solution is thought to be in an equilibrium with a K^+-valinomycin complex. In addition the K^+-valinomycin is in equilibrium with a KCl solution within the electrode. Any change in solution K^+ results in a change in K^+-valinomycin and internal KCl solution within the equilibrium of the electrode body. Such a change gives a change in electrode potential which is directly proportional to the change in external K^+.

A-2 Calculation of Potassium Ion Uptake

The potassium ion electrode was calibrated daily with potassium chloride solutions of 0.1 mM, 1.0 mM, 10 mM and 100 mM concentration. The ionic strength of each solution was fixed at 100 mM by use of $CaCl_2$

(the ionic strength of $CaCl_2$ being three times higher than an equimolar solution of KCl). The pH of each solution was standardized at 7.2 by addition of approximately 1 mM Tes (made pH 7.2 with Tris). The results were used as a check of the slope of the electrode.

When assay buffer was used the KCl concentration was brought to a specified value by addition of a specific amount of KCl, usually 5 or 10 μmoles KCl. The resulting electrode potential was then compared to the predicted electrode potential as a routine check before addition of mitochondria. Following addition of mitochondria and subsequently to that, addition of valinomycin, the change in electrode potential was recorded and the resultant change in potential used to calculate the actual change in extramitochondrial K^+ concentration. The calculation was based on the observed slope of the electrode. If the slope was 50 mv/decade change, a graph of electrode potential vs. K^+ concentration with 50 mv slope was used to determine the actual K^+ concentration change for the observed electrode potential change recorded.

The number of nanoequivalents of K^+ required to register the extramitochondrial K^+ concentration change was calculated and the resulting value expressed as the number of nequiv of K^+ either taken up or effluxed.

The rate of uptake or efflux was determined from the initial slope of the trace. It was calculated by determining the nequiv of K^+ required for the observed change in the fraction of a minute examined. The result was expressed as nequiv/mg protein/min.

Fig. A-1. Slope of Orion liquid-membrane potassium electrode (Model 92-19) with decade increases in concentration of KCl. Ionic strength was maintained constant at 0.1 M with $CaCl_2$ addition where necessary. Standard solutions were adjusted to pH 7.2 with addition of 1 ml of 50 mM TES (adjusted pH 7.2 with TRIS) per 100 ml KCl solution.

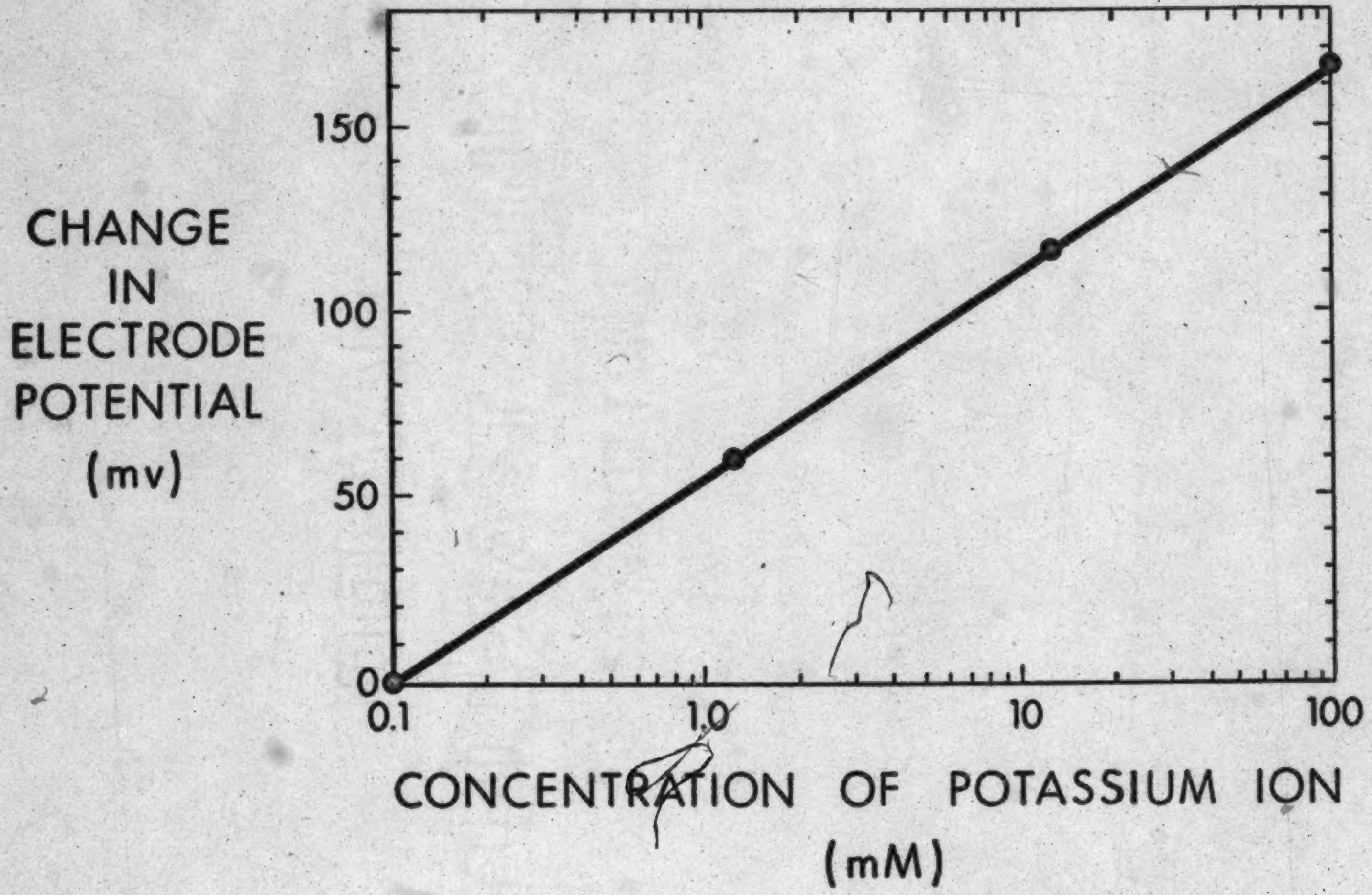
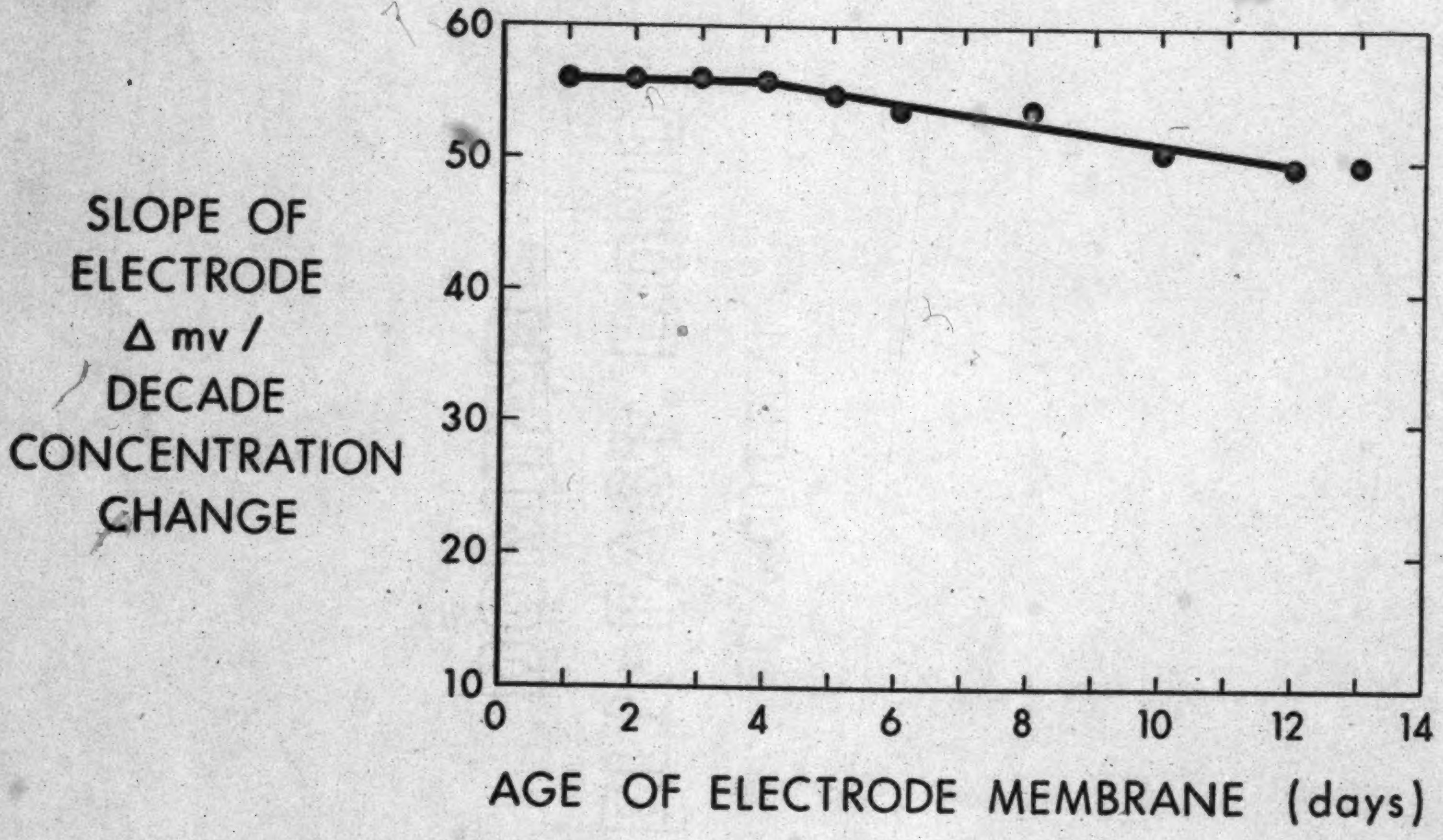

CHANGE IN ELECTRODE POTENTIAL (mv)
0
50
100
150
0.1
1.0
10
100
CONCENTRATION OF POTASSIUM ION (mM)

Fig. A-2. Slope of an Orion Model 92-19 liquid-membrane potassium selective electrode as it changes with age. The slope is a daily average of the change in mv recorded with stepwise increases in KCl concentration from 0.1 mM to 100 mM. Ionic strength was maintained constant at 100 mM with $CaCl_2$.


SLOPE OF ELECTRODE Δ mv / DECADE CONCENTRATION CHANGE
60
50
40
30
20
10
0
2
4
6
8
10
12
14
AGE OF ELECTRODE MEMBRANE (days)

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